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Every company a teaching company

BRITISH industry is in a pretty bad way. Its share of the world market and of the domestic market has declined steadily for many years, and there is no feeling that anything is coming along to save it from even further decline. The per capita output of British industry is only half that of industry in France, Germany, Japan and the United States; every other statistic tells the same gloomy story.

There are a hundred and one remedies offered, many of which are directed at increasing investment or improving labour relations, and in the past few years the Science Research Council (SRC) has itself looked increasingly at the problem of the intellectual input into industry. For it is undeniable that British industry has consistently failed to attract enough bright graduates and PhDs, and has not given enough scope and status to those it has employed. In efforts to stimulate the university-industry links, which seem to come much more naturally in other countries, the SRC and associated government departments have launched a variety of ideas: 'Bosworth' courses, Cooperative Awards in Science and Engineering (the CASE scheme), Total Technology, joint SRC/Social Science Research Council postgraduate training, academic-industrial collaboration schemes, a revamped PhD . . . and now, the teaching company.

In a report just issued (*The Teaching Company*, available from the SRC, free) a joint working party of the SRC and the Department of Industry propose that two or three companies should be added annually to a list of those in which postgraduate students could spend, say, two years working towards a higher degree with a mixture of formal courses and on-the-job projects planned to improve the company's manufacturing operations. Perhaps half a dozen students would be involved in each company; already plans are being made to start experimental operations in four companies. The students will not be doing research in the strict sense—they will be learning about manufacturing technology from practical examples while keeping one foot in a university. Proponents of the teaching company idea freely admit that it is largely modelled on the teaching hospital concept.

The hope is that a corps of well equipped graduates will emerge from the scheme, with experience of running an industrial show and ability to talk to universities about industrial needs in manufacturing technology. These graduates would then start, as one member of the working

party put it, to create oases within the present industrial desert. Those who have passed through a teaching factory should be obvious candidates for posts as production directors, or even managing directors, of companies before too long.

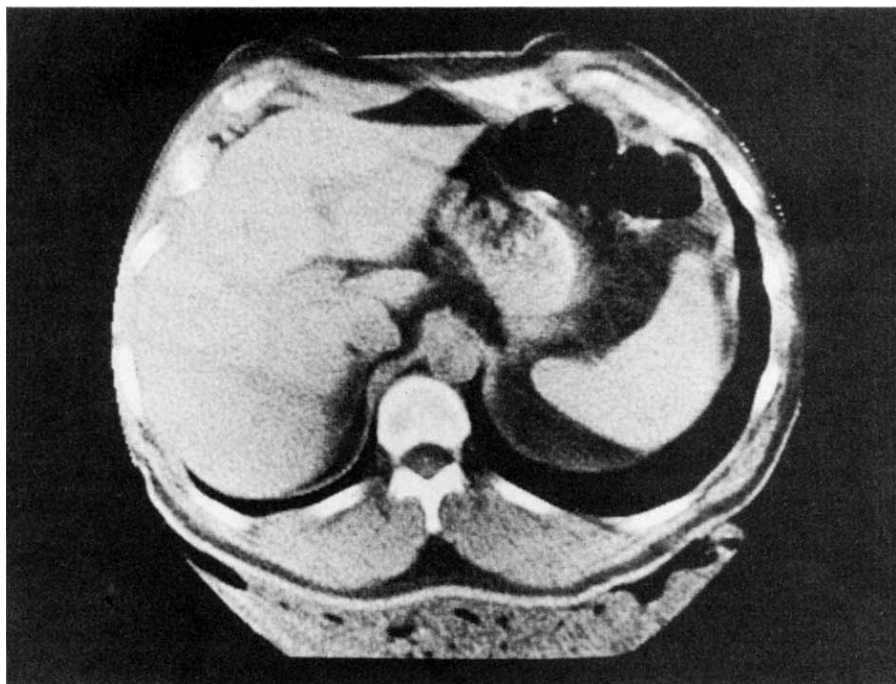
One's first reaction may well be surprise that this sort of thing is not happening already. Surely, in view of all the plans with which SRC have regaled us over the past few years, graduates ought to be moving more freely between university and industry. The problem seems to be that manufacturing technology has always been lowly rated both by hide-bound industry dubious of the need to hire graduates and by universities which find that the subject does not sit too easily among other more academic pursuits. But is the teaching company the answer? There are some serious objections to be considered, and most of these are a matter of scale.

If a mere handful of companies are to be so designated, and these participate voluntarily, indeed willingly, they are already set apart as remarkably enlightened organisations which, quite rightly, probably stand to profit substantially from their involvement.

Embryo manufacturing engineers also need to know about the just-average companies and even the appalling ones. Otherwise there is a danger that on emerging from the programme they will recoil in horror at the general industrial landscape and return to work at the teaching company! There must also be some doubt that even the best run company can provide a continuous flow of good projects to which students can contribute significantly in two years without feeling that the whole thing is being trumped up and is irrelevant to the company's real operations. Finally, many recent initiatives to bring universities and industry closer together have only met with real enthusiasm in new universities; the older ones have viewed innovative programmes as not their concern. Unless the idea of the teaching company can be as acceptable in Oxford as it is in Salford, the proposal will have a diminished impact.

Industry's problems in getting and using graduates are, at root, social ones. Class distinctions, (even within the professions, let alone between workers and management), attitudes of school-children to engineering, the prestige of pure science, anti-intellectualism, pay scales—these all contribute to the bind in which industry finds itself. A handful of teaching companies is not enough. Should not every company be prepared to help train graduates?





Scan of abdomen showing liver, pancreas and spleen (A-scan photograph)

tion of "audenes", analogous to the "phosphenes", may be within reach as well.

Doppler ultrasonic blood flow measurement is now accepted as a useful technique for the vascular surgeon as well as the obstetrician, who uses it to record the foetal heart. Here, the ultrasonic energy is continuously transmitted, unlike the pulsed ultrasound used in echo scanning techniques. The red cell acts as an effective scatterer, and on the assumption that the velocity of the red cell and of the blood as a whole are correlated, it provides a moving target to follow in the vascular system. These meters at present measure blood velocity, but it should be possible to produce quantitative flow meters once the correct method of frequency analysis of the scattered ultrasound is discovered and techniques are developed for measuring the diameter of the artery.

The application of ultrasonic scanning has introduced a new dimension. Small piezoelectric crystals producing 1.5 or 5 MHz ultrasound are coupled by means of oil or water to the skin. The crystals transmit and receive echoes from interfaces separating media of differing acoustic impedance. The third ventricle in the brain is just such a surface, so its displacement, which indicates intracranial pathology, can be measured with moderate ease.

The use of 'A scan' techniques in cardiology for recording the movement of the mitral valve enables the calcification of its leaflets to be assessed by non-invasive means. The separation of the myocardium and the epicardial sac due to infection can also be diagnosed and allows progressive changes to be assessed. The size of the heart and the dimensions of the left ventricle can also be measured. And switched crystal arrays will enable dynamic studies to be made, enabling the volume changes of the ventricle throughout the cardiac cycle to be measured using the techniques developed for angiocardiology. At present, contrast media must be injected and rapid multi-shot X-ray techniques applied.

'B scan' ultrasound has made a significant contribution in gynaecology and obstetrics. Foetal life and growth can now be followed throughout pregnancy, and the placenta can be accurately located. Liver and kidney scans are also useful for the detecting of abnormal pathology.

The recent advance in thermography is also relevant, since the speed and resolution of the latest instruments permits the easy detection of perforating veins and the observation of peripheral vascular changes without the disturbance to the circulation caused by injecting contrast media or radioactive isotopes. It is also of use for

Harnessing electronics to medicine

As world resources are turned increasingly towards ameliorating environmental problems and improving the health of the population, the growth of electronic technology in medicine is well under way. But one of the crucial factors that will determine the advance is finance. Bernard Watson reports on a trend now represented most popularly by the EMI scanner.

ANYONE who has found himself in a hospital at various times since the Second World War could not have failed to notice the increasing sophistication of the equipment used to diagnose ailments and monitor the progress back to health. Modern technology and medicine now work hand in hand, and continue to open up new vistas.

The development of machines for the automatic chemical analysis of blood and new radio-immuno assay techniques for hormones has contributed to a rapid increase in laboratory diagnostic techniques. Ion sensing electrodes will soon be available which will bring back to the bedside the measurements hitherto made in the laboratory; indwelling electrodes, being miniaturised versions of those used for *in vitro* determinations, will be used for continuous monitoring, while semiconductor probes, such as ion sensing field effect transistors, may also become available.

Laboratory measurements like these supplement the measurements made by direct attachment to the patient. Cardioscopes and arrhythmia detectors, which may include computer surveillance of patients, are to be found in the intensive care and coronary care wards, as are automatic respirators.

Indeed, the whole array of life support systems has reached a high degree of complexity.

Therapeutic advances have also been made. The electronic pacemaker is now accepted and even implanted in the aged patient. The artificial kidney has advanced from the early Keel parallel plate dialyser, using cellophane as a membrane, to the disposable capillary kidney, though the cost remains a major disadvantage. This could give way to the wearable kidney with absorbents, or to the development of the storage of donor organs to such a degree of sophistication that matching can take place over months instead of more than 48 hours.

A prosthesis for the patient who has lost a limb will soon include electronic feedback from muscle groups that are still able to signal the brain's commands, provided implantation of the device is accepted. Electronic aids for the blind will also improve. And if the electrical stimulation of the visual cortex to produce significant emanation of light (or "phosphenes") can be harnessed, together with a reduction in size of the solid state camera, the 'artificial eye' will be realised. A prosthesis for the deaf based on the produc-

detecting deep vein thrombosis. Although mammography using xero-radiography may be used for confirmation, the X-ray dose is still higher than that preferred for routine screening purposes, and thermography will be very useful for six-monthly checks.

These are but a few of the electronic devices which are available to the clinician. The most important advances, however, have been in diagnostic imaging. Previously the radiologist struggled for better definition of pathological and functional change in his patient using conventional X-rays with source, patient and plate in simple configurations. Now, the position of the patient and tube and the tube voltage can be more easily manipulated for better definition.

But the outstanding advance in this field has been the X-ray scanner developed by Geoffrey Hounsfield for EMI. Before the advent of this technique, the clinician had to rely on a barrage of techniques to detect intracranial pathology. The techniques depended on the preferential uptake of a radioactive isotope by the tumour, or the experienced eye of the neurophysiologist looking for changes in the EEG. Final confirmation involved an angiogram or an air encephalogram with their attendant risks.

Hounsfield's approach involves the measurement of the absorption of the soft tissue displayed, but its sophistication is that it gives a definition not seen before in radiology. The initial EMI scanner, moreover, which could only be used for the head, has been followed by a body scanner. The disadvantages of standard tomographic procedure are the indistinct picture of the layer under scrutiny and the superimposition of the blurred images of neighbouring layers.

In the body scanner, the patient is X-rayed as a series of 13 mm thick tomographic sections. Each section is examined during each 20-second scanning sequence. The X-ray source and the detector array are mounted on a scanning frame contained in a gantry. A further detector records the intensity of the primary beam. The section is scanned linearly across the patient and the transmission intensity recorded, producing more than 18,000 readings during the scan. At the end of the scan the frame is moved 10° and a further linear scan commences. The sequence is then repeated through a total of 180°.

The readings of intensity are digitised and analysed by a minicomputer to produce the absorption values at 80,000 points within each section. The magnetic store can store eight 13-mm sections. The information for each slice is recorded in the form of a matrix of

square picture elements contained within a circle of 320 picture points. A typical element represents an area in the slice of 1.25×1.25 mm when the machine is set up for a 400-mm body diameter, and 0.75×0.75 mm at the smallest body diameter setting of 240 mm. The absorption values are expressed in units from -500 to +500, with water at 0 on the scale.

As a wide range of absorption values are recorded during the scanning procedure, a control allows a specific range of values to be displayed. The values are permanently recorded, so any range can be displayed on the television monitor. Bulk storage of patient data is readily available using magnetic tape. For the first time accurate measurements of absorption values to $\pm 0.25\%$ are possible with adequate resolution for the analysis of pathological changes in the organ under examination. Clinical assessment of this technique is awaited, but early pictures demonstrate the detail that can be seen even by the inexperienced reader of radiographers.

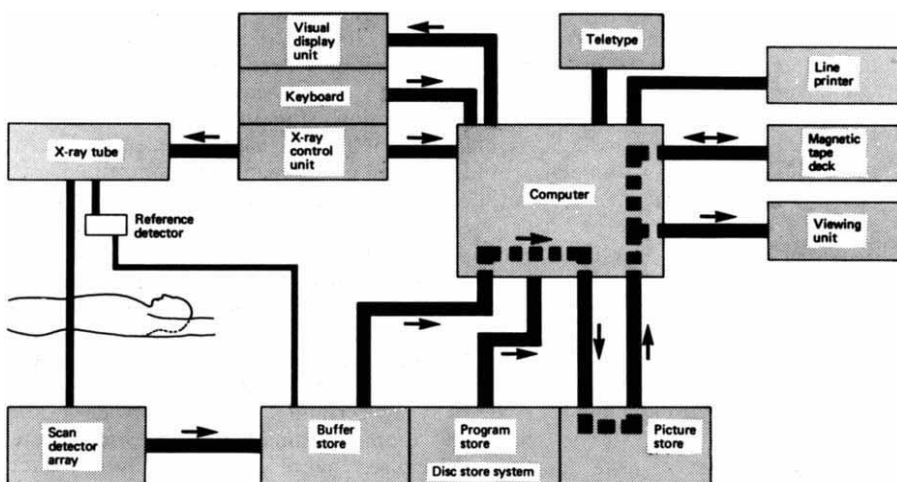
Therapeutic applications must follow this development since the definition in the image gives positional information not previously available. The instruments for non-invasive investigation of most patients is now possible without trauma or risk of death, although the inherent danger of exposure to radiation remains. Better therapeutic techniques, and improvements in preventive medicine, are being sought, but therapeutic advances are not yet keeping pace with diagnostic skills.

If technology can produce advances in therapy and preventive medicine as well as contribute to improved social conditions, the recent advances in medicine will be fully realised and the new diagnostic techniques will produce more than just accurate data for analysing failures. Much, however, depends on the crucial factor of finance. In the UK, research and development in medical electronics is con-

siderable but scattered over many hospitals as well as industrial and government research laboratories with little co-ordination. The Department of Health and Social Security (DHSS) has tried over the past 10 years to encourage specific developments where it has been able to define a need. Pacemakers with ever-greater reliability provide one example and work on these contributed to the development of the miniature nuclear battery for longer-life pacemakers. Companies will generally look for a promising home market before they invest since the overseas medical market is difficult to service and brings its own problems in differing safety standards and clinical approaches. In the case of the initial prototype EMI scanner, finance was provided jointly by the DHSS and EMI.

More could be done if resources were available, but the cost of development is escalating. Development facilities are presently lacking in the hospitals where therapeutic development could be done cheaply and effectively because of the close contact with clinical problems. A capital grant alone will not solve the problems, however. Trained personnel such as engineers and technicians are also needed to use the equipment and integrate it successfully into clinical medicine if there is to be more effective diagnosis and treatment. The development funds of the DHSS have increased more than five-fold during the past 10 years, but the exact level of expenditure is difficult to assess because of the diversity of the programme.

Health expenditure must increase as medical science unravels more of the diversity of disease processes. But the requirement that it be cost effective often causes administrators to wonder whether too much money is devoted to interesting but rare diseases. Animal experimentation might be preferred and it has its place, but it will never surpass carefully collected data from



EMI scanner: schematic diagram of data collection and processing system

sick patients. On the other hand, the clinician has little time to undertake applied research. Considerable improvement in medicine would occur if money could be provided to finance multidisciplinary teams on well-planned

projects in which industry would collaborate by developing the necessary technology.

But at the moment the need to treat more patients than ever before clashes with increasingly rigid budgetary con-

straints. As in so many areas, but particularly in research, a world of priorities means that the capacity to find technological solutions might exist but the economic encouragement often does not. □

I have just returned from attending the Fourth International Conference on the Unity of the Sciences, held at the Waldorf-Astoria Hotel in New York from November 27-30. The subject of the conference was "The Centrality of Science and Absolute Values", and it was attended by some 350 of what the programme describes as "the world's most eminent scholars and scientists" from 60 different countries. Readers of *Nature* will remember the editorial, "Bringing men to the Moon", published on October 25 1974, just before the Third Conference was held in London last November. The editorial questioned the status of the International Cultural Foundation, which was sponsoring (and financing) the conference.

The Foundation was started by the Reverend Sun Myung Moon, who is also the founder of the Unification Church. This church has been severely criticised, mainly for its strong anti-communist views, and for what some consider its undue influence on young converts. The *Nature* article suggested that the participants in the conference might find that they were being used to give respectability to an organisation of which they did not entirely approve. Although the ICF exists as "a non-profit organisation dedicated to promoting academic, scientific, religious and cultural exchange among the countries of the world", critics have suggested that its aims may be less disinterested and more political.

The Fourth Conference included a few representatives from communist-governed states. Invitations had been sent to several people in Soviet Russia, but these were not accepted—an experience not unknown to organisers of the most orthodox scientific gathering. But scientists came from Hungary, Poland and Yugoslavia. In 1974 India was not represented, but this time several Indians attended, as did Egyptians, Lebanese, Israelis, and many others from "third world" countries. As before, we had a galaxy of Nobel Laureates, in physics, chemistry, physiology and other subjects. Several theologians, including Jesuits and other Roman Catholics, as well as members of other sects and different religions, were among the participants. The social sciences were well represented.

Last year the advance announcements about the Third Conference

listed eminent names of supporters and participants, some of whom later dissociated themselves with the organisation. The opinion was stated (in *Nature* and elsewhere) that this might have been done to try to secure wider

Attending a Moon conference



KENNETH MELLANBY

support. I believe that the use of any names without permission was due to misunderstanding—I know one Briton who was approached who said he could not attend but "would help if he could", and then objected when he was listed as a supporter. This year I do not think that anyone was named who was not genuinely willing to help.

Critics of the ICF have queried the selection of those invited, and of the programme of the conference. They have suggested that members may have allowed themselves to be put in a position where they could not freely state their views. Regarding the selection: the invitations came from the ICF, but names were chosen by entirely independent committees of advisers, American and international (I must confess to being one of their number). I do not know of any case in which a name was suggested and the individual was not invited. It is true that the majority of members were middle-aged, and that few strident marxists were included but they certainly included a wide range of opinions and beliefs. Any gaps were caused by refusals, and by the fact that the advisers did not wish to

include those, however well intentioned, who would wreck the event. In fact a few uncompromising critics did attend.

There was no censorship of views or opinions whatever. The 120 or so papers, which were previously circulated to the groups which discussed them, were read by the committee chairman, and sometimes authors were asked to shorten their text, but no matters of substance were excluded. As one who chaired a discussion group I can confirm that no one was muzzled. The conference certainly cannot be criticised as not allowing complete freedom of expression to all participants.

The \$500,000 question remains to be answered: was the conference worth while? I personally think that the answer is "yes". Many equally expensive international gatherings, even those dealing with important scientific topics, are clearly a waste of time and money. Little new is said or heard—"Professor Blank read his paper" (the same one read so often before). Here we had a gathering of good men and women with good minds who seriously discussed the problems facing the world today, and the ways in which science could and should be involved.

I know of no other gathering where this would have been possible. There was less nonsense talked than might have been expected—in my opening address I echoed a warning in last year's *Nature*, when I said that the present disenchantment of the public with science and scientists was "sometimes generated by scientists themselves—when they appear to speak as experts in fields where they possess no expertise".

We attempted to define the boundaries of the various branches of science, and the relations between the natural and social sciences. We discussed the problem of values, in regard to the family, to religion and to morality. Consideration was given to problems facing universities and research institutions, and to the ethics of the application of modern technology. The position of science in resolving world inequalities and in the creation of any future world order was also discussed. We did not solve all the world's outstanding problems, but perhaps we made some progress in the right direction.

international news

Genetic manipulation: recommendations drafted

from Colin Norman, La Jolla

AN ADVISORY committee of the National Institutes of Health (NIH), which met here on December 4 and 5, has drafted a set of strict regulations to control the use of a revolutionary, but possibly hazardous, technique for manipulating genes from living organisms. Its action, which follows several months of controversy and uncertainty, is the latest step in a tortuous process of self-regulation by a segment of the scientific community. Although its recommendations will apply only to research supported by the NIH, they are likely to have broad international implications, because several other countries—including Britain—are also going through the difficult business of trying to draft regulations governing use of the controversial genetic manipulation procedure.

The technique, in short, involves the use of recently discovered enzymes, called restriction enzymes, to rearrange genetic material in combinations which are unlikely to have occurred by natural evolution. For example, it enables genes to be snipped from the DNA of virtually any organism and inserted into a virus or a bacterium in such a way that they will be copied each time the virus or bacterium reproduces (a process known as cloning).

Such direct genetic manipulation has already opened up some exciting areas of research because it offers a relatively simple means of multiplying and studying genes from various organisms, including man. And, more distantly, it may have potential applications in medicine, agriculture and industrial processes, such as correcting genetic deficiency diseases, inserting nitrogen-fixing genes from bacteria into non-leguminous plants so that they would no longer require nitrogen fertilisers, and constructing novel strains of bacteria for such tasks as eating up oil spills.

But the technique also involves some potential hazards, the nature and likelihood of which can only be speculated upon. One worry, for example, is that viruses or bacteria bearing genes

transplanted from another organism could conceivably be endowed with unpredictable biological properties such that, in the worst imaginable case, a novel epidemic could be let loose if they were to escape from the laboratory. A particular problem is that unknown bits of DNA may be transplanted along with the genes under investigation, which opens up the remote possibility that, for example, cancer-causing genes could be introduced into a virus or bacterium and cloned. And such fears are heightened by the fact that most of the experiments are carried out on a laboratory strain of the bacterium *E. coli*, a micro-organism which is a common inhabitant of the human gut.

Although most of the potential horror scenarios are highly speculative, and there are no experimental data to confirm or deny that they are plausible (see box), few scientists are prepared to state that the technique is entirely safe. Consequently, in July 1974, a committee of the National Academy of Sciences, led by Paul Berg of Stanford University, issued an unprecedented call for a voluntary

moratorium to be placed on some uses of the technique, at least until the possible hazards have been evaluated and some regulations have been drawn up. A meeting of some 140 geneticists from various countries was subsequently convened last February at Asilomar, California, to discuss the intricacies of the matter and recommend some guidelines to the scientific community.

The Asilomar conference was faced with a tough dilemma. Although the voluntary moratorium seemed to have been universally respected, there was already some agitation for it to be lifted because it was holding up potentially valuable research; but experience with conventional laboratory safety procedures does not inspire much confidence that viruses or bacteria bearing transplanted genes could be prevented from escaping into the environment, with possibly dire consequences. Many laboratory workers, for example, have suffered infections as a result of research on hazardous pathogens, and the 1972 London smallpox outbreak, which originated in a research laboratory, amply demonstrated how conventional safety procedures can break down.

The Asilomar conference hit on a neat solution to the dilemma, however. It recommended that, in addition to the use of conventional, physical safety precautions, potentially hazardous experiments should only be carried out on viruses or bacteria which have been genetically crippled so that they would be incapable of surviving outside an artificial laboratory environment. It was generally anticipated that such crippled strains could be produced within a matter of weeks, and everybody could soon be safely back at work.

But, unfortunately, some of the optimism turned out to be unfounded, because attempts to develop a crippled strain of *E. coli* have run into numerous snags, and parallel efforts to disable a strain of virus known as bacteriophage lambda, which infects bacteria and



Paul Berg: "happy" with draft

which is a possible vehicle for inserting genes into *E. coli* for cloning, also took longer than anticipated. Until such strains are available, the Asilomar guidelines specify that the voluntary moratorium should be maintained for many kinds of experiments. Consequently, most planned experiments have effectively been under an embargo for 18 months.

It is in that difficult situation that the NIH advisory committee has been trying to write acceptable regulations from the Asilomar guidelines. And it has also been working in an atmosphere charged with suspicion and unconfirmed rumours that embargo-breaking experiments are being carried out clandestinely. In short, the committee has been under considerable pressure to come up with regulations which would allow some of the work to be resumed, and which would at least remove the uncertainty. As one committee member said, if the committee failed to draft regulations at its December meeting, "the dam would be likely to break".

Thus, early in July, a subcommittee under the chairmanship of David S. Hogness, of Stanford University, drafted a set of regulations for consideration by the full committee at a meeting held in Woods Hole later that month. The Hogness version offered some stringent controls which, in most respects, conformed to the Asilomar guidelines, but which were weakened in a few key places at the Woods Hole meeting. It was those changes which have caused the committee most of its problems, and which led to the meeting in La Jolla.

The Woods Hole draft was to have been the committee's final report, but when it was circulated around the scientific community, it encountered so much criticism—including adverse reaction from two committee members, Roy Curtiss III of the University of Alabama, and Stanley Falkow of the University of Washington (who was not present at the Woods Hole meeting)—that it was never published in final form. Instead, the committee chairman, DeWitt Stetten, Deputy NIH Director for Science, appointed another subcommittee under the chairmanship of Elizabeth Kutter of Evergreen State College, Washington, to draft an alternative set of regulations for the committee to consider. The Kutter draft, which was completed in November, was in some respects more restrictive than either the Hogness or Woods Hole versions.

Among the criticisms levelled at the Woods Hole draft was a statement signed by some 50 participants at a conference held in August at Cold Spring Harbor, who complained that it "appears to lower substantially the

safety standards set and accepted by the scientific community as represented by the meeting at Asilomar". And another particularly influential critic of the draft was Paul Berg, who wrote in a letter to Stetten that the restrictions proposed for some types of experiments are "marginal and inadequate considering the risks the document itself concedes"; he suggested that in one particular case, the regulations "are very likely to draw the charge of self-serving tokenism". Some critics took the opposite view, however, suggesting that the Woods Hole regulations were so strict as to constitute unwarranted infringement on academic freedom.

Thus the committee met here in a situation which had all the ingredients of a possible show-down, but it nevertheless conducted its business with little animosity. The entire committee turned up (15 members including the chairman), together with six special consultants who included Berg and Maxine Singer, a virologist from the NIH who was an organiser of the Asilomar conference.

On the first day of the meeting, the committee approved, by a series of close votes, restrictions on some types of experiment which differed little from the Woods Hole draft, and which would inevitably have drawn considerable criticism for being too lax. But on the second day, it went back over some of those recommendations and, by equally close votes, tightened the regulations considerably, so that in some instances they are even more stringent than the Asilomar guidelines.

It is difficult to know exactly what made a few committee members change their votes, but one member indicated privately that after considering the matter carefully, he decided that in cases where there is disagreement, the committee should, as a matter of policy, adopt the more stringent option. It is always possible to lower the restrictions at a later date, he suggested, if evidence becomes available to suggest that the risks have been overstated. Another consideration was the practical one that if the committee adopted regulations which meet with criticisms for being too lax, then it was entirely possible that the matter could be taken out of the hands of scientists, and the technique regulated by legislation—a possibility which clearly bothers many people because it smacks of political infringement on academic freedom.

The committee's move to adopt strict regulations was made easier by an important development. A week or two before the meeting, Roy Curtiss, whose laboratory has been working full-time since the Asilomar conference on the problem of constructing a genetically

Figuring the risk

THE hazards associated with cloning recombinant DNA molecules can only be speculated about, since there is no experimental evidence to prove or deny that they exist. Thus the NIH advisory committee was attempting to write regulations based largely on guesswork about the likelihood that, for example, a bacterium or virus bearing genes transplanted from another organism could pose a danger to animals or plants—a situation, as DeWitt Stetten, the committee chairman points out, "in which Lloyd's of London refuses to write insurance".

The committee therefore unanimously approved a resolution urging the NIH to sponsor a potentially hazardous experiment, in maximum physical containment facilities, to gain some data on the likely extent of the risks. The experiment, which was suggested by Sydney Brenner of the MRC Laboratory for Molecular Biology, Cambridge, would involve splicing genes from a polyoma virus (a mouse tumour virus) into a bacterial plasmid, cloning the recombinant molecule in *E. coli*, and infecting young mice with the modified bacterium.

The idea would be to monitor the mouse's serum to determine whether antibodies to polyoma virus are formed. If they are, it would indicate that the transplanted genes are working, and are being transmitted into the animals' blood stream, in which case there is a real danger that many of the postulated risks exist. If no serum antibodies are formed, it would indicate that at least in this case, the genes are not being expressed in the animal's system, which would suggest that some of postulated hazards are less plausible. There is a danger, however, that such a negative result could be over-interpreted as suggesting that recombinant experiments are inherently safe.

If the experiment is carried out, it would be performed in maximum containment facilities at the NIH, which easily meet the P4 requirements, and it would be conducted by an eminent virologist.

crippled strain of the *E. coli* K12 bacterium, finally produced a strain which seems to meet all the requirements. He has tested it in rats, and found that it does not survive in the intestine, and after some more tests are completed, he believes that it will fit the bill. Similarly, at least two strains of bacteriophage lambda have passed

preliminary tests which indicate that they are crippled sufficiently to provide an extremely high biological safety barrier. Again, they need more rigorous testing before such expectations can be confirmed. The committee was thus able to write regulations specifying the use of crippled micro-organisms for many types of experiment, with a high expectation that the strains would soon be available.

This, in short, is what the committee finally decided. It has defined a series of conventional, physical safety levels, designated as P1 to P4, ranging from simply the use of standard microbiological techniques (P1), to specially designed facilities with negative air pressures, isolated by airlocks, with showers at each exit, and so on (P4). It has also defined three biological safety barriers, designated EK1, EK2 and EK3 for experiments in which genes are inserted into *E. coli* K12 for cloning.

At the risk of slight distortion by over-simplification, the three levels are defined as follows.

- EK1. This is the lowest level of biological containment. It requires use of standard *E. coli* K12, which, according to several tests, is naturally a feeble strain. The genes to be cloned would first be spliced on to either a bacterial plasmid (a ring of DNA which replicates inside the bacterium independent of the bacterium's own DNA) or on to the DNA of bacteriophage lambda which would then infect the bacterium.
- EK2. This level of containment involves the use of crippled strains of *E. coli* or bacteriophage lambda for which laboratory tests have indicated that fewer than 1 in 10^8 cloned pieces of DNA would survive outside the laboratory.
- EK3. This level is essentially the same as EK2, except that it has been rigorously tested by field trials in man, primates or plants.

At present, only the EK1 level is available, but if the crippled *E. coli* produced by Curtiss, or the crippled bacteriophage lambdas (the most promising of which has been produced by Fred Blattner of the University of Wisconsin) meet the requirements, they would constitute an EK2 system. The committee itself will examine the test data and certify whether any of those strains do, indeed, meet the requirements for EK2. The tests needed for EK3 have not yet been undertaken, and they could take several months to complete.

The committee's chief task was to assign various levels of containment to different types of experiments. First, however, it specified some experiments which are considered potentially too risky to undertake at all. The committee recommended, for example, that

DNA from highly pathogenic organisms (class 3, 4 and 5 agents according to the classification adopted by the Center for Disease Control, CDC) should not be cloned, that genes which cause the production of highly toxic compounds (such as diphtheria toxin) should not be spliced on to other pieces of DNA, and so forth. There was little disagreement over the need to outlaw such experiments.

The sharpest areas of disagreement involved the assignment of so-called shotgun experiments to various safety levels. Shotgun experiments involve chopping up an organism's whole genome (or at least a substantial part of it) and splicing the fragments into plasmids or into the DNA of a bacteriophage for insertion into *E. coli*. Such experiments are considered to be hazardous since they involve cloning unknown fragments of DNA, and those involving DNA from primates or man are considered the riskiest of all since the cloned DNA may contain genes from cancer viruses. The committee's final recommendations are contained in the accompanying tabulation; they are

mostly more stringent than the Woods Hole draft, and they are all at least as strict as the Asilomar guidelines.

At least one novel concept emerged from the discussions on shotgun experiments. It was agreed that since embryonic cells are less likely to have been infected by viruses, DNA taken from embryonic tissue is likely to be safer to use in a shotgun experiment than DNA from adult tissue. Thus, such experiments were assigned a lower category. Moreover, it was also agreed, after considerable discussion, that if the DNA to be cloned has been purified so that at least 99% of it is a known, non-harmful gene, the cloning can be performed at a lower physical containment level.

Another area which caused considerable discussion was the containment levels necessary for a class of experiments involving the splicing of DNA from various organisms on to the DNA of animal viruses, and infecting cell cultures with the resulting hybrid virus. In other words, the virus is used as a vehicle for cloning the transplanted genes. Two different viruses, a monkey

THE committee defined four levels of physical containment, designated, in order of increasing severity P1 to P4, and three levels of biological containment, EK1 to EK3 (see text) and assigned experiments to them on the basis of potential risk. The following is a tabulation of the committee's proposals showing containment levels for various sources of DNA. It is an unofficial version which should be checked against the committee's final report when it is published.

a. Shotgun experiments using <i>E. coli</i> as the host	
Non-embryonic primate tissue	P3 + EK3 or P4 + EK2
Embryonic primate tissue	P3 + EK2
Other mammals	P3 + EK2
Birds	P3 + EK2
Cold-blooded vertebrates, non-embryonic	P2 + EK2
embryonic	P2 + EK1
Invertebrates and lower plants	P2 + EK1
Higher plants	P2 + EK2
Higher plants which produce toxins	P3 + EK2
Prokaryotes which exchange genes with <i>E. coli</i>	
Class 1 pathogens (for example, enterobacteria)	P1 + EK1
Class 2 pathogens (for example, <i>S. typhi</i>)	P2 + EK2
Higher risk pathogens	Banned
Prokaryotes which do not exchange genes with <i>E. coli</i>	
Non-pathogens	P2 + EK1
Low risk pathogens	P3 + EK2
Moderate pathogens	P3 + EK3 or P4 + EK2
Higher pathogens	Banned
In all above cases, if the DNA is at least 99% pure before cloning, the physical containment level can be reduced one step.	
b. Cloning virus genes in <i>E. coli</i>	
Animal viruses	P3 + EK3 or P4 + EK2
Plant viruses	P3 + EK1 or P2 + EK2
c. Eukaryotic plasmid or organelle DNA cloned in <i>E. coli</i> if DNA is 99% pure, and consists of harmless genes	
Primate tissue	P3 + EK1 or P2 + EK2
Others	P2 + EK1
If DNA is impure, then shotgun conditions apply	
d. Animal virus vectors	
Defective polyoma + DNA from class 1 virus (CDC classification) or non-pathogen	P3
Defective polyoma + class 2 virus	P4
if virus DNA known to be non-harmful	P3
Defective SV40 + class 1 virus or non-pathogen	P4
Defective SV40 + non-pathogenic DNA from either prokaryotes or eukaryotes	P3
Defective SV40 or polyoma lacking late genes + prokaryotic or eukaryotic DNA provided no virus particles are present	P3

virus called SV40 and a mouse virus called polyoma virus, are being used in such experiments.

The committee eventually assigned all such experiments to very high physical containment facilities, according to a set of guidelines drawn up by a group of animal virologists, including Berg and Singer, who were present at the committee meeting as consultants or observers. Berg apologised for bringing in such complicated proposals at a late stage in the proceedings. Another committee member reflected that such last-minute proposals indicate the speed with which the field is moving, and that he would like to keep it moving, a statement which Berg labelled "self-serving".

After the meeting was over, Berg said that he was happy with the final draft, and indicated that it had tightened the restrictions in areas which

bothered him in the Woods Hole recommendations. He said that he believes the final recommendations "have incorporated the spirit of Asilomar".

Although the committee has demonstrably accepted strict regulations, it is nevertheless open to the charge that its deliberations have had no input from the general public. The committee consists entirely of scientists, some of whom are using, or planning to use, the cloning technique, a fact which has already raised suggestions of conflict of interest. But the stringency of the regulations is such that those criticisms can easily be blunted.

The committee's report should be available in a few weeks. The recommendations will go to the Director of the NIH, who will probably accept them, and they will eventually be used by the NIH in awarding grants for such research. □

Concorde Mark II?

WITH Concorde due to commence passenger services on January 21, and a decision awaited from the US Government clearing it for the Atlantic run, the prospect of a "second-generation" supersonic airliner was aired last week by the Vice-chairman of Rolls-Royce (1971), Mr Kenneth Wilkinson, when he delivered the annual Wright Memorial Lecture to the Royal Aeronautical Society in London.

Concorde, he said, was expected to demonstrate the value of supersonic air transport for a sufficiently important segment of the market to justify a second-generation version for service in 10 to 15 years' time. The indications were that such an aircraft could be produced profitably with "acceptable noise characteristics and economic operation at less than first-class fares".

ENERGY needs for Egypt's projected population of 220 million in the year 2100 would demand the construction of two Aswan High Dams a year; water requirements demand the equivalent of two extra Nile Rivers, making desalination essential. Only nuclear energy offers the means to fulfil these demands. The agreement signed with Washington during the recent visit of President Sadat to the United States involves two light water power reactors, using boiling water if Westinghouse is brought in or pressurised water if General Electric is.

The International Atomic Energy Agency (IAEA) and the General Egyptian Electricity Corporation (GEEC) estimate that Egypt will be using nuclear energy to cover 50% of its energy needs for electricity and desalination by the year 2000 and will need 20,000 tons of uranium concentrate for the reactors—the equivalent of 400 million tons of oil.

Cumulative uranium requirements for power and desalination programmes were found to be in the range of 19,000–25,000 tons in case of a heavy water reactor (HWR) strategy, and 30,000–40,000 tons for a light water reactor (LWR) strategy. In the year 2000 alone, the demands are expected to be 2,000–2,500 tons and 3,200–4,000 tons, respectively.

These needs make intensive uranium exploration, coupled with a suitable reactor policy, essential to the development of a local nuclear fuel cycle technology. These matters are now under study, but the first atomic power plant (EFAPP), together with the two American reactors which will be constructed at Sidi Kreir near Alexandria and

should be operating in 1982, will be of the LWR type. No final decision on subsequent nuclear power stations has been taken.

Estimates of the water demands for agricultural, municipal and other purposes suggest a deficit of 2×10^9 – 3×10^9 m³ by 1980, increasing to 17×10^9 – 18×10^9 m³ by 2000. Nuclear desalting would make up part of the shortfall—about 4×10^9 – 5×10^9 m³—in the year 2000. The power requirements will be around 9,000 MWe.

Letter from Egypt

from Salah Galal, Cairo

This means a nuclear programme 50% greater than that projected by the GEEC for nuclear power is needed.

● Ahmed Sultan, Egypt's Minister of Electricity visited Paris recently to discuss the purchase of two more nuclear reactors, which will be in addition to the two American reactors. During last week's state visit to Egypt of the French President, Valéry Giscard d'Estaing, the two countries signed an agreement providing for the construction of a 600 MW nuclear power plant in the Nile delta area. The plant is due to go into operation in 1984.

● The Animal Bioclimatology Unit of the Egyptian Atomic Energy Establishment has decided to take part in agriculture and livestock projects which might help remedy deficiencies in the country's food production. It is believed that radioisotopes can be used to improve productivity in hot desert areas:

Professor Taymour Kamal, who heads the unit, has used radioisotopes and tracer techniques to develop new methods for predicting heat tolerance in farm animals. Young animals selected for hot areas would be expected to have high productive potential after growing in the Sahara's hot climate. Kamal has criticised conventional heat tolerance indices as unrelated to the productive performance animals, and says his tracer methods measure directly the animal's loss in body fat and lean mass.

One of his methods is based on a 3-minute count of radioactive ⁴⁰K occurring naturally in the animal's body before and after exposing it for 3 days to a temperature of 32 °C. The loss of lean tissue can then be calculated since the radioactive potassium exists in a definite and constant ratio to all natural potassium, which is found in fairly constant concentration in lean tissue. Young animals which show little or no tissue destruction during the heat exposure are considered heat tolerant and suitable for raising in hot areas.

A second tracer method measures both the lean tissue loss and the fat loss. This is based on a determination of the total body water of the animal before and during the 3 days of heat exposure. These figures are subtracted from the corresponding live body weights to obtain the total body solids, mostly fat and protein, before and during heat stress. The animals screened have shown great variation in the percentage loss of body solids ranging from 0.2% to 24.5%. Those animals which show a small loss are considered heat tolerant. Other tracer methods for heat tolerance are still under investigation in the unit.

Mr Wilkinson gave a figure of £5,500 millions for development costs. The aircraft would sell for about £65 millions, and use a turbofan derivative of the Rolls-Royce Olympus 593 engine presently powering Concorde. But, he added, there should be only one design. This would mean another joint international venture, which might include the Soviet Union as well as the USA.

Rolls-Royce meanwhile successfully completed a £100 millions deal at the weekend with the Chinese National Technical Import Corporation under which China will, over the next two years, import a limited number of Spey turbofan engines manufactured in Britain. Rolls Royce will supply the Chinese with the expertise they need to manufacture the engines under licence. The licence itself covers both military and civil versions of the turbofan, and Rolls-Royce are understood to be supplying afterburners along with the complete engines. With China already heavily committed to the Hawker Siddeley Trident, which is fitted with Spey engines, the reinforcement of the link with British aerospace technology encouraged speculation about the status of options China has on three Concorde.

● European cooperation on another project, the Multi-Role Combat Aircraft (MRCA), received a bit of a boost with the signing late last week of a contract for the initial investment required to start production. The contract, worth several million pounds, was signed by the NATO Military Management Agency (NAMMA), which runs the programme for the three governments involved, West Germany, Italy and the UK. It authorises a three-nation airframe consortium to start work. The production go-ahead itself, for more than 800 aircraft, is expected next year. □

Pollution proposals

by Allan Piper

A PROPOSAL for blanket legislation to standardise pollution levels within the European community was modified last week when EEC environment ministers meeting in Brussels reached a compromise which will allow member states some flexibility in the administration of pollution controls. Instead of observing pollution limits based on measurements of waste levels at the source of emission, members will be able to opt for a scheme whereby pollutant concentrations are monitored after dispersion into the environment. In considering each case on its individual merits the scheme will allow some countries to dump more toxic waste than others.

The agreement represents something of an achievement for the UK Government which, with the backing of its Royal Commission on Environmental Pollution, had opposed the Commission's proposals for uniform standards. With an alternative available, the UK can continue its present policy of using "quality controls" to restrain the dumping of toxic wastes like cadmium and mercury.

The wrangle between the UK and its EEC partners began when the environment ministers last met in Luxembourg in October. The UK then rejected the Commission's call for the introduction of so-called 'emission norms'—uniform standards based on the levels of toxic waste actually produced from any source. The UK argument in favour of quality controls was based on the view that EEC member states had diverse environments which could accommodate widely differing concentrations of pollutants. The northern Atlantic off the Scottish coast, the argument ran, was more easily able to disperse a given level of toxic substances than the coastal waters of the Adriatic.

The latest agreement, which came as a surprise, means that while the Commission will stand by its original proposal for emission norms, individual members can opt for quality control. But there is a caveat. They must satisfy the Commission that their controls are equally effective in maintaining levels below the prescribed limits. The UK Department of the Environment is confident that Britain will have no difficulty in meeting this condition.

The quality controls will be re-examined by the Council every five years. Meanwhile the finer details of the two systems will be drafted. The Commission has two years to complete its proposals which will then go before the Council for approval. Once approved, the member states will begin arrangements to implement the necessary legislation. Since this will probably take about five years altogether, the UK's present pollution policy is unlikely to come under further EEC scrutiny for about a decade.

The main opposition to the idea of control by quality norms came from West Germany and Holland, both of which have to contend with the peculiarly acute pollution problems posed by the Rhine. Their antagonism apparently stemmed from their concern that favourably located countries like Britain should not gain unfair commercial advantages by not being required to bear the individual cost of complying with uniform legislation. UK industry could have been faced with a bill estimated at £750 millions had the original community proposal remained unmodified. □

Kovalev sentenced

from Vera Rich, London

ON the very day that he was due to receive his Nobel Peace Prize, Dr Andrei Sakharov was in Vil'nyus unsuccessfully seeking admission to the trial of Dr Sergei Kovalev, a biologist and member of the banned Moscow group of Amnesty International.

Dr Kovalev was arrested a year ago on various charges of "anti-Soviet agitation and propaganda" and "slandering the Soviet Union". These included the production and distribution of *samizdat* journals and of Solzhenitsyn's *Gulag Archipelago*, and also the circulation of the illegal *Chronicle of the Lithuanian Catholic Church*, which provided the reason for holding the trial in Vil'nyus rather than Moscow. Dr Kovalev is not a Lithuanian, nor, so far as is known, has he any connection with the Catholic Church. His only connection with the *Chronicle* seems to have been that when his flat was searched, a few pages from a Russian-language edition of it were found among his collection of *samizdat* materials.

If it was hoped that by changing the venue the trial might attract less notice, this was largely vitiated by Dr Sakharov's presence, which helped to ensure that world attention was focused on the Vil'nyus courtroom. Also present in Vil'nyus, and unable to gain admission to the courtroom, was physicist Yuri Orlov, a friend of both Kovalev and Sakharov. Kovalev was sentenced to seven years' jail with hard labour and three years' exile.

During the Vil'nyus hearing the case was recalled of another dissident, the Ukrainian mathematician Leonid Plyushch, held in a penal psychiatric institution on account of his dissident and "separatist" views. Plyushch has been a cause celebre for all those who have campaigned against alleged misuses of psychiatry for political ends. Recently, after a massive rally and campaign in France, in which left-wing and communist organisations played a considerable part, the Soviet authorities promised Mrs Plyushch that her husband would shortly be set at liberty. No definite information as to a release date has been given, but according to dissident sources, one of the psychiatrists who had been treating Plyushch was called to give evidence against Dr Kovalev and stated that he had lied when he wrote that dissidents were being confined in mental hospitals as a means of political repression.

Meanwhile Mrs Sakharov has reportedly criticised the western press for emphasising the difficulties of Soviet intellectuals instead of the idea that "an innocent man ought to have nothing to fear in our country". □

correspondence

New president of Soviet Academy

SIR,—In your recent article "Appointment to Soviet Academy threatens autonomy" (December 4, page 377), your correspondent's interpretation of the changes in the Academy leadership was superficial and prejudiced. The conclusion that the replacement of Academician V. A. Kotelnikov by Academician A. P. Alexandrov indicates "a greater degree of Government supervision of the Academy's work in future" was made, first, because Alexandrov was introduced at the Academy meeting by Politburo member M. A. Suslov and not by somebody from the Academy's own ranks, and, second, because Alexandrov himself is a member of the Central Party Committee Plenum.

Both aspects are irrelevant in any assessment of the Academy's future. The replacement had to be made by a high Party or Government official because the Academy is not and never has been independent; it is a State scientific establishment.

With the position of the chairman of the "Ideological Commission"—which includes the Party's department on science and education—vacant more than a year already, M. A. Suslov (as top Party ideologist) is now the highest official responsible for science and education. There is also nothing sinister in the fact that Alexandrov is a member of the Central Party Committee. The most liberal Russian poet, writer and editor Alexander Tvardovsky was also a member of the Central Party Committee.

I consider that the election of Alexandrov now is a concession from the Government, a move to meet the demand of the Academy for a more popular and prominent figure. If this is so, the new election is a liberal decision, and does not reflect an increase of political pressure. Moreover, regarding Alexandrov himself, it should be noted:

- When, during Khrushchev's time, classical genetics was still forbidden and Lysenko reigned supreme, the only place where large-scale, serious genetic research was possible was the big biological division of the Kurchatov Institute of Atomic Energy headed by Alexandrov—a division created specifically for confrontation with Lysenko pseudogenetics.

- When in 1966 Alexander Solzhenit-

syn decided to give public readings from his banned novels, the only place where he was able to do this was the Kurchatov Institute. The hundreds of scientists there warmly welcomed the writer, and Alexandrov was reprimanded for taking this liberty.

- When in 1970 I was put by force into a mental hospital for political reasons, academicians who acted on my behalf, strongly and voluntarily, included Alexandrov. Indeed, he was the most influential among them, and the only one who did not know me personally, yet he spoke strongly in support of my immediate release.

- Alexandrov's predecessors, Kotelnikov and M. V. Keldysh, signed a letter to *Pravda* on August 29, 1973, against A. Sakharov. Alexandrov did not.

The Soviet Academy, being a state body, has never really been autonomous. But it has moral authority and political influence which derives mostly from the personal prestige of academicians and their scientific stature. It also stems from the courage of some of the Academy's best people in their struggle for scientific integrity and scientific and human rights. This struggle has not always been successful, but there have been several cases in recent years when the majority of the Academy has strongly opposed Government pressure on certain issues.

Your correspondent was also wrong to predict a short term for Alexandrov merely because of his age. The position of president is not tenured for life; it is subject to re-election every five years. The only president who was not able to complete this minimum term was the young and bright Sergei Vavilov, who died from a heart attack in 1951.

Yours faithfully,

ZHORES A. MEDVEDEV

National Institute
for Medical Research,
London, UK

Multiplying ventures

SIR,—During the past fortnight I have received three communications on the formation of new groups. The first of these is from the Society of Experimental Biology; it refers to the formation of a neurobiology group. The second one comes from the secretariat of a newly formed European Neuroscience Association. The third one is a communication concern-

ing the first meeting of a newly formed European Society of Neurochemistry.

I am already a member of the International Brain Research Organisation (IBRO) and I believe I am also a member of the International Society for Neurochemistry. I also frequently attend meetings of the Neurochemistry Group which meets under the auspices of the Biochemical Society.

Faced with this plethora of new ventures one is tempted to ask the organisers: "Is your venture really necessary?" It seems to me as if in this field there is a development of much overlap. Is there no roof organisation that could be consulted when such new societies are formed? The European Neuroscientists' missive contains the threat that "a fee structure should be set up by the first Council for individual membership". The European Neurochemists require a registration fee of not more than £15 for participant members of their first meeting.

For one whose interests happen to bridge the areas of several or all of these bodies considerable monetary expenditure seems to be involved. Surely this multiplication of organisational effort is not in the spirit of the times.

Yours faithfully,

H. BLASCHKO

University Department of
Pharmacology,
Oxford, UK

Journal guidelines

SIR,—Why does Professor Ziman think it a populist folly to disclose the names of referees to authors? In the *Journal of Aerosol Science*, 1 (1), 1970, I wrote "A feature of the new journal is that no cloak of mystery will shroud those who accept, reject or accept subject to modification the papers which are submitted to it. There are three reasons for this unorthodox exposure of the referee. First, the authoritarian age is over, second, the standard of critique will be raised and lastly, it is hoped, encouragement and benefit will result to authors."

Six volumes of editorial experience confirm this statement. What has Professor Ziman to offer in support of his assertion?

Yours faithfully,

C. N. DAVIES

Department of Chemistry,
University of Essex, UK

news and views

Chemical identification of a natural opiate receptor agonist in brain

from Leslie Iversen

MORPHINE and related opiates interact with highly specific receptor sites which are present in greatest abundance in certain circumscribed areas of brain, particularly in association with neural pathways involved in the perception of pain (see Snyder, *Nature*, **275**, 185; 1975). None of the established chemical transmitter substances in brain seems to interact strongly with the opiate receptors, suggesting that the mode of action of these drugs does not involve any of these known mechanisms. This has led to renewed interest in the hypothesis that the brain may contain a hitherto unidentified substance which normally acts as the endogenous activator of the opiate receptors, a possibility which now appears to have been substantiated.

Hughes (*Brain Res.*, **88**, 295; 1975) and Hughes *et al.* (*Life Sci.*, **16**, 1753; 1975) obtained evidence for the existence of an endogenous factor with the properties of morphine in brain by testing brain extracts on two biological preparations that were known from previous studies to respond specifically to opiate drugs. These tests are based on the ability of morphine and related drugs to block electrically evoked contractions of mouse vas deferens or guinea pig intestine. In each case a crucial item of evidence was that the effects of morphine and of the morphine-like compound in brain extracts could be blocked by low concentrations of specific morphine antagonists such as naloxone. Using these test systems as bioassays, it was possible to develop an extraction and purification scheme for the isolation of pure material with the properties of morphine from pig brain. This substance was called "enkephalin" and had a molecular weight of approximately 1,000; it rapidly lost biological activity if exposed to proteolytic enzymes, suggesting that it might be a peptide. A similar substance could be shown to be present in extracts of cow, guinea pig, rabbit, rat and mouse brains. In the pig brain, enkephalin was unevenly distributed, being most concentrated in regions known from previous work on other species to contain the highest density

of opiate receptor sites.

Another method for detecting the presence in brain extracts of a substance acting like morphine is to measure its ability to displace the specific receptor binding of radioactively-labelled opiate drugs such as etorphine or naloxone to brain membrane preparations *in vitro*. This approach has been used successfully by Terenius and Wahlstrom (*Acta physiol. scand.*, **94**, 74; 1975) and by Pasternak, *et al.* (*Life Sci.*, **16**, 1765; 1975), who have also described the existence of a morphine-like substance in extracts of calf, rabbit and rat brain. The compound has a molecular weight of approximately 1,000 and is susceptible to degradation by proteolytic enzymes, suggesting that it may be a small peptide. It has a regional distribution in brain similar to that previously observed for opiate receptor sites. Terenius and Wahlstrom (*Life Sci.*, **16**, 1759; 1975) also obtained evidence for the existence of a similar substance in samples of human cerebrospinal fluid. The potencies of morphine agonists (mimetic drugs) in the radioactive ligand binding assays are decreased by the presence of low concentrations of sodium ions in the incubation medium, whereas the potencies of antagonists are increased by sodium ions (Pert and Snyder, *Molec. Pharmacol.*, **10**, 868; 1974). The ability of the morphine-like factor to displace radioactive ligands was greatly decreased by adding sodium chloride suggesting that the substance is a relatively pure agonist at opiate receptors.

A very important advance in this field is now described by Hughes *et al.* (page 577 of this issue). They have been able to complete the chemical identification of enkephalin previously purified from pig brain. Using conventional methods of amino acid sequence analysis and independent mass spectrometric analysis they report that porcine enkephalin consists of a mixture of two pentapeptides: H-Tyr-Gly-Gly-Phe-Met-OH (Met-Enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-OH (Leu-Enkephalin), in a ratio of approximately 3:1. Both peptides have now been synthesised, and the syn-

thetic materials shown to mimic the actions of naturally occurring enkephalin on the guinea pig ileum and mouse vas deferens preparations. Met-Enkephalin is effective at very low concentrations on the mouse vas deferens, in which it is about twenty times more potent than the powerful opiate agonist normorphine. It is also some three times more potent than morphine in a ligand binding assay. Leu-Enkephalin is somewhat less potent than the methionine analogue.

It remains to be seen whether enkephalin as described by Hughes *et al.* corresponds to the endogenous morphine-like factors of Terenius and Wahlstrom and Pasternak *et al.* although this seems very likely. The relationship, if any, of these small peptides to the somewhat larger peptide described by Teschemacher *et al.* (*Life Sci.*, **16**, 1771; 1975) and by Cox *et al.* (*Life Sci.*, **16**, 1777; 1975) in extracts of bovine pituitary glands is also obscure. These authors reported that impurities in commercial preparations of the pituitary peptide ACTH and a substance present in extracts of pituitary had acted like morphine in the guinea pig ileum, mouse vas deferens and ligand binding assays. These findings may perhaps relate to the observation of Terenius *et al.* (*Eur. J. Pharmacol.*, **33**, 395; 1975) that various synthetic ACTH-like peptides have properties similar to morphine. It is possibly significant that the amino acid sequence of Met-Enkephalin is also found in residues 61-65 of the pituitary peptide β -lipotropin, which is related to ACTH.

The identification of enkephalin and its availability in synthetic form will allow rapid progress to be made in answering the many questions that remain. Does enkephalin have analgesic properties? (As a corollary, is enkephalin even capable of penetrating into the brain from the bloodstream after systemic administration?) Why are there apparently two peptides with similar biological properties? Are they always found together in different brain regions? Perhaps the most important practical question is whether

enkephalin offers the long sought approach to the development of a non-addictive analgesic. Kosterlitz and Hughes (*Life Sci.*, **17**, 91; 1975) have described some preliminary evidence in favour of this view. They point out that enkephalin has some unusual pharmacological properties resembling those seen in certain recently developed synthetic drugs of the benzomorphan class, which seem to be free of addictive properties in monkey tests. These properties are, first, a relatively rapid rate of onset and short duration for the effects on opiate receptors in isolated test organs and second that the actions are less readily blocked by standard antagonist drugs such as

naloxone. To antagonise a response of given magnitude requires about a four times higher concentration of naloxone when enkephalin is the agonist than when conventional agonists such as normorphine are used.

There will also undoubtedly be rapid progress in establishing radioimmunoassay methods to measure enkephalin in brain, and possibly immunohistochemical techniques to elucidate its precise cellular localisation in different brain regions. This information should help to clarify the possible physiological role of enkephalin as a neurotransmitter or neuromodulator substance associated with pain pathways in brain. □

A photosynthetic structure

from A. G. Lee

READING the literature on photosynthesis is almost certainly bad for the health. To be told that in the green photosynthetic bacteria, exposure to light causes a flow of electrons from Chl-660 to Chl-770 then to P840 and finally to C551, C553 and C555 does little to reassure one, especially when informed that Chl-770 is also called B810, that P840 could be an aggregated form of Chl-770 and that C551 and C555 also masquerade under the names C419 and C422 respectively. These names of course reflect the fact that the physical and chemical identity of the species are generally unknown, and that they have been recognised only from changes in absorption spectra. For any satisfactory model of photosynthesis, it will be necessary to translate these labels into actual chemical species present within the photosynthetic system. In a paper by Fenna and Matthews in this issue of *Nature*, (page 573), a start is made towards this goal.

In the green bacterium *Chlorobium limicola* (once called *Chloropseudomonas ethylica*) the photosynthetic apparatus is contained within egg-shaped vesicles, about 50 nm wide and 150 nm long, arranged as a layer along the inside face of the cytoplasmic membrane. In this it differs from the purple photosynthetic bacteria, where the photosynthetic membrane is continuous with the cytoplasmic membrane. The photosynthetic apparatus in the green bacteria also differs in that they contain an extrinsic chlorophyll-containing protein which is water soluble and can be removed by changes in buffer strength and pH: the chlorophyll-containing proteins in purple

bacteria and plants are firmly attached to membranes and can only be removed by detergent treatment. Because it is water soluble, it has been possible to purify and crystallise this chlorophyll-protein from *C. limicola*, and it is the structure of this protein that is reported by Fenna and Matthews.

Although the bulk of the chlorophyll in *C. limicola* is chlorobium chlorophyll (also called bacteriochlorophyll or Chl-660), there is present as a minor component bacteriochlorophyll (Chl-770, also called, you will remember, B810), and it is bacteriochlorophyll that is contained in the water-soluble protein. Since the amino-acid sequence of the protein is unknown, the X-ray crystallographic studies only give information about the α -carbon backbone, but the structure is sufficiently defined to show the basic protein conformation.

The protein consists of three identical subunits, each containing in its centre seven bacteriochlorophyll molecules arranged in an apparently irregular fashion. The structure of each subunit is described as a distorted hollow cylinder, with one end of the cylinder possibly being open. The interesting and unique aspect of the structure is the hollow cavity within each cylindrical subunit, with the bacteriochlorophyll molecules being bound to the 'walls' of this cavity. The binding of the bacteriochlorophyll molecules to the protein is partly due to interactions between the phytol chains and what are probably hydrophobic residues facing towards the centre of the subunit. There are also, however, extensive interactions involving the Mg ions of the porphine rings, either through hydrogen bonding between the protein

and a water molecule coordinated to the Mg, or through a direct interaction between the Mg and a suitable coordinating group of the peptide. Hydrogen bonding is also possible between some of the substituents of the bacteriochlorophyll and the protein. Although there seems to be no direct contact between the porphine rings, the average centre-to-centre nearest neighbour distance is only 12 Å, so that efficient energy transfer is possible within each subunit.

If the structure of this particular chlorophyll-containing protein is at all typical, then it makes the derivation of molecular models from absorption spectra seem an almost impossible task, since although the planes of the seven porphine rings in each subunit are close to being parallel, there is no obvious pattern to their relative orientation. The question as to how important are the chlorophyll-proteins generally in the photosynthetic process still remains open however. The actual role of the bacteriochlorophyll-protein for example is still uncertain: although it has been suggested that it acts to transfer excitation from the antenna chlorobium Chl-660 to the reaction centre P840, it has not yet been demonstrated that photosynthesis fails to occur in its absence. The fact that it is water soluble also makes it highly atypical. The isolation of chlorophyll-containing proteins from other photosynthetic systems requires the use of detergents, and it is not then at all clear whether the isolated complexes correspond to species actually present in the membrane. Thus although membrane-bound proteins are generally isolated in detergent solutions as lipid-protein complexes, this does not mean that they were present in that form in the membrane: the presence of an organised lipid bilayer in the membrane means that hydrophobic surfaces of the membrane proteins are isolated from the aqueous phase, whereas in the detergent solution, a specific lipid-protein interaction is necessary to provide the same protection. The observation that the majority of the chlorophyll in a photosynthetic membrane is found associated with protein after detergent treatment similarly does not mean that it was the same in the membrane. In fact, it is known that chlorophyll incorporated into a lipid bilayer in the absence of any protein will adopt an aggregated structure analogous to that proposed for antenna chlorophyll (Strouse, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 325-328; 1971). Nevertheless, it is obvious that chlorophyll in the reaction centres must be closely associated with protein, and the structure reported by Fenna and Matthews shows something of the complexity that might be expected. □

Site-directed tumour chemotherapy

from a Correspondent

THE interesting idea of site-directed killing of tumours by cytotoxic agents coupled to molecules with affinity for the target tissue was first suggested seventy years ago by Paul Ehrlich. Apart from the obvious and probably surmountable difficulty of obtaining suitable tissue-, or better tumour-specific agents (antibodies for example) for use in treatments of this kind, there is the problem of getting to the cancerous site sufficient of the cytotoxic substance to cause an effect. Exceptionally potent toxins are required therefore that can kill cells when present in very low concentration. Diphtheria toxin is one such agent. The toxin is a protein of molecular weight 62,000 which is synthesised in high yield by certain lysogenic strains of *Corynebacterium diphtheriae* after infection with bacteriophage. This killing effect is mediated by the strong inhibition of protein synthesis, probably by the catalytic addition to aminoacyl transferase II of ADP-ribose from NAD. Since protein synthesis in a fairly wide range of cells is inhibited by the toxin *in vitro*, the fact that for example the growth of mouse L cells is not prevented, means that the cytotoxic action requires binding of the toxin to surface receptors as an obligatory first step. The toxin may be cleaved by some proteases into two polypeptide fragments A and B. The B fragment binds to cell surface receptors and fragment A inhibits polypeptide elongation. Receptors are present on human cells but not mouse cells and although these are very few (25–50 per cell) this is sufficient to make the cells sensitive. Perhaps as few as 1 or 2 toxin molecules delivered to the cell surface are cytotoxic.

Earlier experiments (Moolten *et al.*, *J. natn. Cancer Inst.*, **49**, 1057–1062; 1972; Philpott *et al.*, *Surgery*, **73**, 928–935; 1973) showed that diphtheria toxin coupled to antibodies directed against dinitrophenyl or mumps virus selectively killed cells bearing these antigens when the appropriate conjugate was added to the culture medium. *In vivo* the toxin-anti-dinitrophenyl antibody conjugate protected to some extent hamsters concurrently challenged with viable DNP-coated sarcoma cells. In their latest paper Moolten *et al.* (*J. natn. Cancer Inst.*, **55**, 473–477; 1975) have applied sublethal doses of diphtheria toxin conjugated to antibodies directed against SV40-associated cell surface antigens, to hamsters bearing SV40-induced tumours with some encouraging results. Treatment of ham-

sters with a single dose of the hamster antibody-diphtheria toxin conjugate prolonged the mean survival time of animals challenged concurrently with SV40-induced sarcoma cells and delayed the appearance of the tumour after transplantation. The conjugate, somewhat disappointingly, had no effect on established tumours of the SV40 fibrosarcoma but did cause regression of an SV40-induced lymphoma. The conjugate therefore seems to be more effective against the slow growing lymphoma than a rapidly proliferating tumour such as the fibrosarcoma, probably because of the appreciable delay in the entry of toxin molecules into cells and inhibition of protein synthesis after the initial binding to cell surface receptors. Faster growing tumours may replace damaged cells so successfully that overall growth is unimpaired.

The experimental design of these preliminary studies suffers from the use of whole toxin since the molecule, even when conjugated to antibody, still binds to cells through its own specific receptors. This undoubtedly contributes to the more or less severe secondary effects of therapy with toxin-antibody

conjugate, such as damage to non-tumour cells and localised necrosis of subcutaneous tissue at the sites of injection. More positively, the toxicity of the toxin itself may be a useful adjuvant in the therapeutic action of toxin-antibody conjugates since this toxicity appears, to some extent, to be selective for tumours (Iglewski and Rittenberg, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2707–2710; 1974). Certainly, further effects along these lines would be worthwhile using perhaps a proteolytic C-terminal peptide fragment of diphtheria toxin that, although somewhat unstable, nevertheless carries almost the full toxic activity of the whole molecule when tested *in vitro* but cannot bind to surface receptors and therefore has no effect on intact cells lacking surface antigens recognised by anti-tumour antibody.

In another approach to a rational cancer therapy, R. Arnon and M. Sela and their colleagues (*Cancer Res.*, **35**, 1182–1186; 1975) have coupled an anti-tumour antibiotic, daunomycin, to antibodies directed against tumour-associated surface antigens. The difficulty is to prepare covalent complexes that preserve both the antibody activity

Core material at Earth's surface?

from Peter J. Smith

THE idea that the Earth's crust contains rock which once formed part of the upper mantle has become commonplace, especially since the work of Gass (*Nature*, **220**, 39; 1968) and others on the significance of ophiolites. Claims to have recognised material from the core, by contrast, are very much rarer. In view of the greater distances involved, the paucity of core derivatives at the Earth's surface is hardly surprising. On the other hand, modern concepts of mass flow in the mantle suggest that the transport of material from the core region to the crust should at least be possible; and it is to just such concepts that Bird and Weathers (*Earth planet. Sci. Lett.*, **28**, 51; 1975) now appeal to explain the existence of josephinite along Josephine Creek in Oregon.

Josephinite is a rare rock containing a high proportion of nickel (55–75%) and iron (20–45%). In this respect it resembles, and has often been confused with awaruite, a somewhat less rare iron-nickel mineral associated with low-temperature reduction reactions occurring during the serpentinisation of ultramafic rocks. But a detailed petrographic and X-ray study of josephinite and awaruite carried out by Bird and Weathers shows that

the two materials have significant differences. Chief among these is that josephinite contains lamellae and blebs of magnesium silicate and andradite garnet. Some of the least altered samples also contain elemental silicon and a natural equivalent of the artificial substance $\text{CaO} \cdot 2\text{FeO}$, neither of which has previously been found in terrestrial rocks, least of all in awaruite.

The unique composition and structure of josephinite suggests to Bird and Weathers that, in contrast to awaruite, this material has a high-temperature and high-pressure origin, perhaps by crystallisation from an iron-nickel melt. This in turn suggests that the source could lie in the vicinity of the core-mantle boundary. And in the light of modern ideas, the most obvious way of transporting material from the core-mantle boundary to the upper lithosphere would be to have it carried upwards by mantle plumes and emplaced in the crust by means of associated diatremes. For as Bird and Weathers point out, "the distance of vertical transport from the core-mantle boundary region to the surface is certainly no more surprising than the lateral changes of distances established by oceanic anomaly patterns for the present plate population".

and the pharmacological activity of the anti-tumour drug. The most successful method developed by the Israeli group is periodate oxidation of the sugar moiety of daunomycin to generate aldehyde groups capable of forming Schiff base linkages to free amino groups on the antibody molecules. These linkages are then stabilised to secondary amines by sodium borohydride reduction. About 2 to 5 drug molecules could be substituted into each antibody molecule in a form with acceptable capacity for killing cells. The drug-antibody conjugates showed immunological specificity in the sense that conjugates containing antibody directed against different murine lymphoid tumours bearing specific surface antigens were cytotoxic only against the appropriate tumour or tumours bearing cross reacting surface antigens. It should eventually be possible to test *in vivo* the effectiveness of antibody-directed tumour cell killing by daunomycin, particularly as methods are improved for the preparation of tumour-specific antibodies. □

Nuclear shock waves

from P. E. Hodgson

THE concepts of classical physics can often be used to gain a useful insight into nuclear phenomena, especially when relatively large numbers of high energy nucleons are involved. An example of this is provided by what happens when two nuclei collide at high energy. In the region where they first interact the density rises suddenly, and if the relative velocity of the nuclei is greater than the rate of propagation of this density disturbance through the nucleus a nuclear shock wave develops.

The rate of propagation of the shock wave is the velocity of sound in nuclear matter and this can be estimated from the known nuclear compressibility (*Nature*, **254**, 480; 1975) to be that corresponding to an energy of about 10 MeV per nucleon for the colliding nuclei. Such energies can be attained by many heavy ion accelerators so it is possible in principle to produce nuclear shock wave phenomena.

The development of such shock waves has been calculated by Scheid, Mueller and Greiner (presented at Nashville Conference, 1974), and they find for ^{16}O - ^{16}O collisions that even for centre-of-mass energies of 100 MeV densities almost twice normal appear. For energies of 1,000 MeV, densities five times normal occur, and in each case a shock wave is expected.

The shock wave itself is essentially a sharp discontinuity in the nuclear pressure, density and velocity, and it is essential for its application to nuclei

that the thickness of the shockwave front, or region of rapidly varying properties, is small compared with the nucleus itself. The thickness is approximately the same as the mean free path of the nucleons in the nucleus, and as this is rather less than 1 fm, whereas nuclei typically have diameters of a few fm, this condition is expected to be satisfied.

The shockwave phenomena occurring when two spheres of matter collide have already been worked out for stars, and have been applied to nuclei by Wong and Welton (*Phys. Lett.*, **49B**, 243; 1974). For simplicity they considered the collision of two slabs of nuclear matter in one dimension and used the Brueckner equation of state to calculate the density and energy of the nuclear matter in the shock wave as a function of the Mach number, the ratio of the velocity of the shock wave to that of the nuclear sound. They found that the shock wave propagates along the collision axis and that dissociation occurs at a Mach number of about two. Thus we expect to find showers of nucleons emitted in the forward and backward directions.

If one of the colliding nuclei is much lighter than the other, the shock wave front is conical, with the semi-angle of the cone given by the ratio of the shock-wave and particle velocities, just like the electromagnetic wavefront in the Cerenkov effect. Figure 1 shows a schematic illustration of the development of such a shock wave when an ^{16}O ion collides with ^{238}U at energies of more than 10 MeV per nucleon. Such waves are emitted at a definite angle that can be related precisely to the energy of the incident particle. If such an effect were observed in nuclei it could provide evidence of nuclear shock waves.

An attempt to detect nuclear shock waves in energetic heavy ion interactions has been made by the Berkeley group (Crawford *et al.*, *Phys. Rev. Lett.*, **34**, 329; 1975) by bombarding

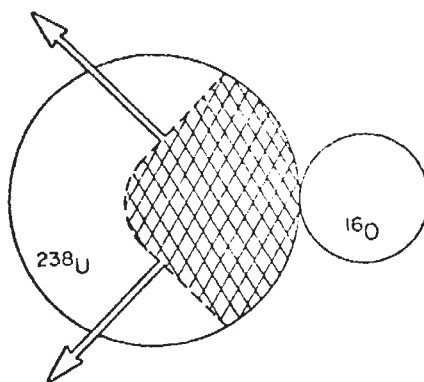


Fig. 1 Schematic illustration of the development of a shock wave front after the collision of an ^{16}O ion with a ^{238}U nucleus.

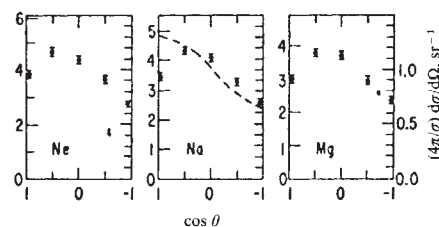


Fig. 2 Angular distribution of fragments from uranium irradiated by 28 GeV protons. The dashed curve refers to carbon emitted from uranium bombarded by 5.5 GeV protons, which shows no peaking.

gold nuclei with 25 GeV ^{12}C ions. At such high energies, corresponding to velocities about a fifth of that of light, one might expect a nuclear shock wave to develop. They measured the angular distribution of fragments with charges from 5 to 9 and energies up to 1,000 MeV emitted from the nuclear disintegrations and looked for the peak around 50° that would be the definitive sign of a nuclear shock wave. The data did indeed show a slight maximum at this angle, but it was not statistically significant and cannot be interpreted as evidence for a shock wave.

Another attempt to detect nuclear shock waves has been made recently by Remsberg and Perry at Brookhaven National Laboratory (*Phys. Rev. Lett.*, **35**, 361; 1975). They irradiated gold and uranium with 28 GeV protons and looked at the energy spectra of the emitted fragments with charges between 6 and 12. As shown in Fig. 2, the spectra of some of the heavier fragments show a broad peak around 70° ; for lighter fragments the peak shifts to smaller angles and becomes less distinct. These peaks occur at about the angles predicted for a nuclear shock wave, and may indicate the initiation of some kind of collective motion directly associated with the cascade of particles initiated by the incoming proton. More precise data would however be needed to establish the presence of a nuclear shock wave.

Another possible phenomenon that could occur in a nuclear shock wave is the production of what are called ultradense nuclei. These are stable, abnormally dense nuclei in which the energy of compression is compensated for by pion condensation. Such nuclei are at present purely theoretical conjectures, based on our present understanding of pion and nucleon forces. It is further conjectured that such nuclei might be formed in a nuclear shock wave, where the nuclear density may be raised two or more times above its normal value. This might be just enough to cause the nuclei, or perhaps fragments emitted from the energetic interactions, to condense to the ultradense state.

A search for ultradense nuclei has recently been carried out by another

group at Berkeley (Price and Stevenson, *Phys. Rev. Lett.*, **34**, 409; 1975). They bombarded lead with relativistic argon ions having 1.1–1.6 GeV per nucleon, and looked for emitted particles of Z greater than 20 that might be interpreted as ultradense nuclei. No evidence for such particles was found.

There is thus no definite evidence at present either for nuclear shock waves or for ultradense nuclei, but they both provide a stimulus for more theoretical and experimental work, and show the continuing usefulness of classical ideas even in nuclear physics at high energies.

Managing Britain's forests

from Peter D. Moore

THE development of forestry in Great Britain is sheer economic good sense, or so the speakers at a recent conference on Forestry Management sponsored by the Royal Society of Arts in London would have us believe. Our current demand for wood and wood products in Britain is 0.84 m³ per head of population and of this 0.76 m³ is imported. Our total per capita forest resource amounts to only 0.03 ha, about a quarter of that enjoyed by, for example, West Germany. Of this resource, well over half is currently under private ownership and management. Undoubtedly world consumption of timber will continue to rise and our very considerable dependence upon imported timber will place us in a very vulnerable economic position. This fact alone seems ample justification for the expansion of our forestry, as an investment for the future.

The situation, however, is not simple. We live in a society in which the Chancellor of the Exchequer can pledge himself to the support of "a mixed economy in which the private sector is vigorous, alert and profitable", yet this Conference revealed severe discontent amongst the private sector of forestry concerning the proposed system of taxation. Under this, the owner of a forest is not only liable to pay income tax, but also may be liable to the proposed wealth tax and his timber, on harvesting presumably after his decease, will be subject to capital transfer tax. These represent serious disincentives to the potential private forester and evidently demand the close attention of those responsible for the creation of taxation systems. It is surely mindless cynicism, on the part of some, to have regarded afforestation as a technique for tax avoidance.

The British national forestry body, the Forestry Commission, has recently redefined its objectives, presumably out of deference to the vociferous environ-

mental lobby in this country, so that it is no longer simply committed to the production of timber, but also to the "protection and enhancement of the environment and the provision of recreational facilities". One can forgive the foresters a degree of resentment regarding the insertion of these phrases since, as one speaker laconically remarked, no one has asked that the North Sea oil rigs should be painted in aesthetically pleasing colours. More people are likely to encounter forests than oil rigs, however, which places greater social responsibility upon the forester, but one must recognise that forest modification in the interests of amenity costs money or, more precisely, cuts profitability.

If forestry is to provide an amenity it may necessitate planting slower growing species and in such situations that overall productivity is reduced. The provision of amenity may also involve the Forestry Commission in permitting greater freedom of access to forests, which brings the attendant dangers of damage and fire. Undoubtedly one can also anticipate an increased demand for camping facilities, for hunting and angling and possibly for motor rallying. Not all such activities are compatible with one another; there are limitations to multipurpose uses of forest.

The relationship between British forest management and nature conservation is even more complex. It was recognised at the Conference that afforestation of upland moorland generally causes an increase in the diversity of wildlife inhabiting the area, especially during the early stages of forest growth. The replacement of deciduous by coniferous forest in lowland situations, however, has the reverse effect. Even in the uplands the maturation of forest usually brings about a final reduction in plant and animal diversity and many of the species of open moorland habitats will obviously be lost. What received little emphasis in the Conference was the justification for nature conservation. One received the impression that wild plants and animals, their study and conservation, belonged to the amenity of forest use. Although there is a measure of truth in this, it is far from the full story. Steele pointed out that many communities once destroyed could never be recreated, so the maintenance of these fragments of woodland which approximate to our native forest is particularly urgent if we are to have a yardstick against which to measure our achievements in the management of planted forests for timber production. Added to this there is that crucial argument for conservation based upon our limited genetic resources. The loss of a

species of animal or plant, or even the loss of a local population of a species, involves genetic depletion. The consequences of any such loss cannot be calculated, but the repercussions could be economic as well as aesthetic, for one can never tell when a given species will assume economic importance to man.

From the point of view of nature conservation, the most efficient land use practice is the maintenance of habitat diversity alongside the afforestation programme. This attitude was admirably illustrated by the efforts of Lord Dulverton at his estate near Fort William, Scotland. He has adopted a mixed land use policy retaining moor-



A hundred years ago

THE Secretary of the Interior, in his annual report to the President of the United States, commends in high terms the work of the Geological and Geographical Survey of the Territories, and presents the following brief summary of the results for the season of 1875:—The survey under Dr. Hayden continued its labours of the two preceding years in the Territory of Colorado . . .

. . . The districts explored in the past season were not so mountainous as those of the previous years, but were quite remote from settlements, and in perhaps the most inaccessible regions of this continent. The total area surveyed is about 30,000 square miles, portions of which were very rugged. Much of this area is drained by the Colorado river, and is mainly a plateau country cut in every direction by deep gorges or canons, the sides of which show, for geological investigations, admirable sections of the strata forming the earth's crust. The topography of the district surveyed was elaborated in detail by the aid of the plane-table. The exploration of the remarkable prehistoric ruins of Southern Colorado, glimpses of which were obtained the preceding season, was continued with great success. They were traced down the canons to the Colorado river in New Mexico, Utah, and Arizona, and their connection established with the cliff cities of the Moquis of the latter Territory. Hundreds of cave-dwellings, of curious architecture and many miles from water, were found in the sides of the gorges, and the ruins of extensive towns discovered in the adjacent plains, indicating the former existence of a people far more numerous and advanced in the arts of civilization than their supposed descendants of the present day. Of these ruins many interesting sketches, plans, and photographs were made, and a valuable collection of flint weapons, earthenware and other specimens, was gathered.

from *Nature*, 13 (December 23), 156; 1875.

land on upper slopes and combining forestry with improved pasture for sheep on the lower hills. Added to this he stressed the need for maintaining any existing areas of native deciduous woodland, as a genetic and aesthetic rather than an immediate economic investment. Such an attitude of compromise whether on the part of the Forestry Commission or private land-owners would be welcomed by all conservationists. □

Plasticity of the nervous system

from Shin-Ho Chung

A Royal Society Meeting for Discussion, organised by H. B. Barlow and R. M. Gaze, was held on December 4 and 5 at the Royal Society, London.

FOR two days at the Royal Society, thirteen neurobiologists presented recent evidence dealing with the adaptive nature of the nervous system. Although the topics discussed ranged from the amphibian nerve-muscle preparation to the mammalian cortex, the problems posed by new discoveries converged on one central question: what are the underlying mechanisms that enable synapses in the nervous system to be changed in response to an altered external environment? This turns out to be an enormously difficult question to answer.

The simplest system in which plasticity of synaptic connections can be demonstrated is the nerve-muscle junction. It has been known for many years that muscle fibres, when deprived of their original nerves, accept newly formed branches of foreign nerves with which they do not normally connect, but the fate of such aberrant synapses when the cut nerve regenerates is under dispute. R. F. Mark (Australian National University) presented new evidence supporting his earlier claim that 'incorrect' synapses are suppressed when 'correct' synapses are reformed, rather than both remaining active. He suggests that nerve fibres compete for the available synaptic sites, and, if two nerves innervate one target cell, the less preferred one is rendered inactive. Because such a mechanism may account for several puzzling facts noted in the central nervous system, Mark's observations need to be carefully re-examined. The phenomenon of collateral sprouting, as described above, is also known to occur in the mammalian brain. G. Raisman (National Institute for Medical Research, Mill Hill) reported that vacated synaptic sites in the septal nucleus and in the superior

cervical ganglion can be re-occupied by other nerves. When the vagal or hypoglossal nerve is directed to a denervated ganglion, vacated synaptic sites are occupied by the new nerve, each fibre subserving about the same number of sites as the original one.

Raisman's observations clearly demonstrate that specificity for afferent-efferent coupling in the nervous system is not rigid, and under certain conditions a foreign axon is as competent in occupying synaptic sites as the original one. If synaptic coupling between an axon and a target cell is not rigidly stipulated, it is conceivable that an axon may form a large number of aberrant contacts in the course of embryonic development. P. D. Wall (University College London) argued that even in the adult, axons have a far wider anatomical distribution than post-synaptic physiology would suggest. Wall provided evidence that an axon entering the cat spinal cord traverses and forms synaptic contacts over several spinal segments. The distant connections are normally suppressed, but their presence can be unmasked by stimulating the axon electrically. The mechanisms by which such synapses are suppressed remain unknown, as are the means by which post-synaptic cells recognise appropriate axons. There are known to be several regions in the nervous system where an axon forms a series of transient connections during development before establishing stable contacts with post-synaptic cells. M. J. Keating (National Institute for Medical Research, Mill Hill) adduced that the fibres connecting the two optic tecta in *Xenopus* shift their connections continually during development. Such re-adjustments are necessitated by the gradual movement of the eyes relative to the animal's head; in order to register an image seen by the two eyes onto the same area in the tectum, a series of synaptic contacts are temporarily formed and subsequently broken. He presented elegant evidence that the ability of a nerve fibre to reform new connections declines slowly with age. This discovery may be a neurophysiological basis for human learning.

A large part of the meeting was devoted to the mammalian visual system. Anatomical studies have shown that optic axons terminate in bands of cells in the geniculate body, fibres from each eye ending in alternating layers. All the axons originating from the geniculate body project onto the fourth cell layer of the visual cortex, where alternating clusters of fibres subserving one eye are interdigitated with those of the other, giving the appearance of a chequerboard pattern. From his spectacular series of anatomical studies, P. Rakic (Harvard University) elucidated the sequence of

events leading to the formation of these intricate anatomical arrangements in the rhesus monkey. Cells in the different visual centres divide mitotically at different embryonic stages; those forming the geniculate body, for example, are all generated within a few days, whereas the cortical cells are continually being formed over a period of two months. Before the geniculate cells are segregated into several distinct layers, optic nerve fibres invade the region, those from one eye intermingling with those from the other. Similarly, fibres receiving the inputs from the left and right eyes do not form regularly spaced clusters but are distributed as a continuous sheet in the visual cortex. From such amorphous masses of cells and fibres, the ordered patterns of fibres carrying inputs from the right and left eyes gradually evolve.

Because visual fibres from both eyes project to the cortex, most post-synaptic visual cells respond to stimulation of either eye. When an animal is reared with one eye closed, cortical cells then respond predominantly to the eye which has been left open. C. Blakemore (University of Cambridge) showed that in the kitten only a brief period of sensory deprivation during the critical stages causes all cortical cells to be heavily dominated by the normal eye. Evidence that such an ocular dominance, once patterned, is not modifiable by extensive retraining of the deprived eye later in the animal's life was provided by K. -P. Hoffman (University of Mainz). S. LeVay (Harvard University) demonstrated the anatomical basis of ocular dominance in a rhesus monkey deprived of vision in one eye for a year. The alternating clusters of terminals from the right and left eyes in the cortex are no longer of equal size but instead clusters from the normal eye gradually expand, and those from the deprived eye shrink.

There may indeed be a common basis for all the diverse facts put forward at this meeting. If we can unravel the underlying mechanisms by which the visual centres become ordered so exquisitely, we will then understand how terminals there expand and shrink as a result of monocular visual deprivation. Similarly, if the means by which nerve cells recognise synaptic contacts as appropriate or inappropriate are elucidated, it will also become clear how nerve cells make continual adjustments and re-adjustments of their synaptic relations during development. These are problems for which neurobiologists cannot even begin to formulate questions. Answers will be difficult to find when one does not even know what questions to ask. □

articles

Chlorophyll arrangement in a bacteriochlorophyll protein from *Chlorobium limicola*

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The three-dimensional structure of a chlorophyll-containing protein has been determined by X-ray crystallography and shown to consist of three identical subunits, each containing a core of seven bacteriochlorophylls arranged in an irregular fashion and enclosed within an envelope of protein.

THE three-dimensional structure of a bacteriochlorophyll protein has been determined by X-ray crystallography. This is the first chlorophyll-containing protein for which the structure is known, and provides new information on the arrangement of chlorophyll *in vivo*. The bacteriochlorophyll protein is seen to consist of three identical subunits, each containing a core of seven bacteriochlorophyll molecules arranged in an irregular fashion and enclosed within an envelope of protein.

Green photosynthetic bacteria contain small amounts of a water-soluble bacteriochlorophyll protein which can be extracted in aqueous salt solutions without the use of detergents^{1,2}. This complex has been shown to mediate in the transfer of excitation energy from the antenna pigment chlorobium chlorophyll to the photochemical reaction centres of the organism²⁻⁴. The complex from *Chlorobium limicola* has been shown by chemical studies to have a molecular weight of about 150,000 and to contain 21 ± 2 molecules of bacteriochlorophyll *a* (Bchl *a*), tightly, though not covalently associated with the protein⁵. Analyses of the absorption and circular dichroism spectra, recorded at 77 K, gave evidence for interaction between at least five molecules of Bchl *a* (ref. 6). Preliminary X-ray studies of two different crystal forms of the Bchl protein have shown it to be a trimer consisting of three identical subunits⁷. The form in space group $P6_3$ with unit cell dimensions: $a=b=112.4$ Å and $c=98.4$ Å, has one subunit per asymmetric unit and was used for the X-ray structural studies reported here. In this paper we describe the arrangement of the Bchl *a* molecules within the complex as deduced from a 2.8-Å resolution electron density map. Description of the X-ray structure determination will be confined to essential features and a comprehensive analysis will be presented elsewhere.

Structure determination

Details of the procedure used for growing large crystals of the Bchl protein have previously been described^{7,8}. Diffraction data from the native crystals and isomorphous heavy atom derivatives were recorded photographically using an Elliott rotating anode X-ray generator and Enraf-Nonius precession cameras. Integrated intensities were measured from conventional precession photographs by means of a

computer controlled Optronics rotating drum microdensitometer⁹. After symmetry averaging and merging the intensities from 23 different film packs, each set of data reduced to about 16,500 unique reflections, excluding the Friedel related measurements. This total includes 90% of the data to Bragg spacings of 2.8 Å together with 42% between 2.8 Å and 2.75 Å spacings. Excluding the weakest reflections, the mean error in amplitude on scaling together planes within the native and derivative data sets ranged from 2.8 to 3.4%. The crystals contain about 70% by volume solvent, and presumably for this reason do not diffract strongly, particularly at high angles. The resolution of 2.8 Å adopted for this study is the highest which could reasonably be attained using Buerger precession photographs.

Isomorphous heavy atom derivatives were prepared by soaking crystals of the native protein in solutions of heavy atom compounds in 0.01 M Tris-HCl buffer containing 1 M NaCl and 10% w/v $(\text{NH}_4)_2\text{SO}_4$ at pH 7.8. Two suitable derivatives were obtained by soaking in the presence of 5×10^{-4} M potassium chloroplatinate and saturating concentrations of methyl mercuric iodide, each for 7 d. A third, double derivative, was made by soaking in a solution containing both 5×10^{-4} M potassium chloroplatinate and an excess of methyl mercuric iodide, again for a period of 7 d. One heavy atom site in each of the two single derivatives was initially located from a difference Patterson synthesis of the $hk0$ zone. Additional sites in the mercury and double derivatives were then found from projection and three-dimensional difference Fourier maps in the usual way. Further refinement of the heavy atom parameters was carried out using a lack-of-closure least squares refinement procedure applied to a subset of the three-dimensional data, selected to include all of the centric data together with 25% of the acentric reflections¹⁰. The heavy atom parameters used in the final phase calculation are listed in Table 1.

"Best" phases were calculated by the multiple isomorphous replacement method including contributions from measurements of anomalous scattering of the heavy atoms¹¹⁻¹³. The mean figure of merit of 0.57 is probably a conservative estimate since the value of 0.66 for the average r.m.s. lack of closure over E indicates that the E values used had been overestimated. A comparison of the mean heavy atom scattering with the E values (Table 1) shows that the single site platinum derivative contributes substantially less to the phasing than do the mercury and double derivatives. This is not surprising in view of the difficulty in measuring the comparatively small changes in intensities produced by the introduction of a single platinum atom into an asymmetric unit of this size. The Fourier calculation was performed using a fast Fourier transform

Table 1 Heavy atom parameters

Derivative	Z	x	y	z	B	N	$\langle f_h \rangle$	E	R (%)
Potassium chloroplatinite	28.2	0.113	0.443	-0.251	27.5	5,445	44	35	52
Methyl mercuric iodide	40.3	0.379	0.582	-0.007	25.1	5,444	75	38	47
	16.7	0.301	0.439	-0.104	34.6				
	9.3	0.279	0.434	-0.143	9.9				
Double derivative	31.4	0.113	0.443	-0.250	35.0	5,460	85	43	52
	38.7	0.380	0.582	-0.006	25.9				
	14.1	0.301	0.437	-0.104	32.8				
	10.0	0.278	0.434	-0.142	16.4				
	9.6	0.510	0.204	-0.397	23.8				

Z, relative occupancy of heavy atom site in arbitrary units; x, y, z, fractional coordinates; B, isotropic temperature factor ($\text{\AA}^2$); N, number of reflections included in the refinement. $\langle f_h \rangle$, E and R are respectively the mean heavy atom scattering, the lack of closure error and the residual for the centric reflections to a resolution of 2.8 $\text{\AA}$.

program provided by L. F. Ten Eyck¹⁴. The electron density distribution was sampled at intervals of 0.73 $\text{\AA}$ ($c/135$) normal to the c axis and the contour levels traced on to Dayco plastic sheets stretched on to aluminium frames, suitable for observation in an optical comparator^{15,16}.

Description of the molecule

We have suggested previously that in the $P6_3$ crystal form the trimers are positioned on the threefold rather than the 6_3 axes of the unit cell⁷. The electron density map clearly confirms this conclusion. The packing arrangement gives rise to extensive channels of solvent 112.4 $\text{\AA}$ (the a cell dimension) apart and approximately 62 $\text{\AA}$ in diameter centred on the 6_3 axes and running parallel to the c axis throughout the crystal. Each trimer extends 57 $\text{\AA}$ along the threefold axis and has a maximum diameter of 83 $\text{\AA}$ at right angles to it. Thus, the overall dimensions of the trimer correspond closely to those deduced earlier from considerations of symmetry and molecular packing. It was possible to define the inter-subunit boundary by making only one

cut through contoured electron density. The subunits are, however, packed very closely together and extensive interactions between them undoubtedly occur at the interfaces. In contrast, the contacts between one trimer and its neighbours in this crystal form are very tenuous.

The amino acid sequence of the Bchl protein has not yet been determined; it was therefore necessary to rely solely on the continuity of the electron density in attempting to determine the course of the polypeptide chain. By placing markers in the electron density map at probable α -carbon positions it was possible to follow extensive sections of the protein backbone. In some regions, however, particularly in loops on the surface of the molecule, breaks in the continuity do occur and at this stage we are unable to define a unique course for the whole of the polypeptide chain. But, apart from a few ambiguities, the continuity is generally quite good, and is sufficiently defined to give an overall view of the protein conformation. The total number of amino acid residues in each subunit, based on the number of marked α -carbon positions is 327, although it is probable that some residues have been missed in regions where the electron density is not well defined. Assuming a mean residue weight of 110, calculated from the amino acid composition³, the minimum molecular weight of each subunit, including Bchl, becomes 42,300, and for the trimer 127,000.

The conformation of the polypeptide chain is illustrated in Figs 1 and 4, and can be seen to form a distorted hollow cylinder. In our present interpretation of the electron density map, one end of the cylinder seems to be "open", but is occluded by the carboxyl-terminal helix of an adjacent subunit of the trimer. It is also possible to interpret the electron density map in the vicinity of this inter-subunit contact in such a way that a single subunit would form a "closed" hollow cylinder. At present we cannot distinguish with certainty between these two possibilities. The hollow cavity within the cylinder contains the Bchl aggregate. The area of the cylindrical wall which is exposed to the solvent in the trimer is composed almost entirely of 15 strands of β sheet which seem to be mainly anti-parallel. The side of the cylinder in contact with an adjacent subunit has a more complex structure, consisting of four short lengths of α helix interspersed with regions of irregular conformation.

Bacteriochlorophyll core

Within the core of each subunit the electron density clearly corresponds to an array of chlorophyll molecules. Disks of density indicate the positions of the porphyrin rings, whereas the phytol side chains can be seen as resolved ribbons of density. Seven Bchl molecules pack within one subunit, corresponding to a total of twenty-one for the trimer, in agreement with the chemical studies³. The chemical structure of Bchl *a* is known (Fig. 2), and differs from chlorophyll *a* in having two extra hydrogen atoms in ring II and an acetyl group in place of the vinyl group on ring I. The absolute configurations at the

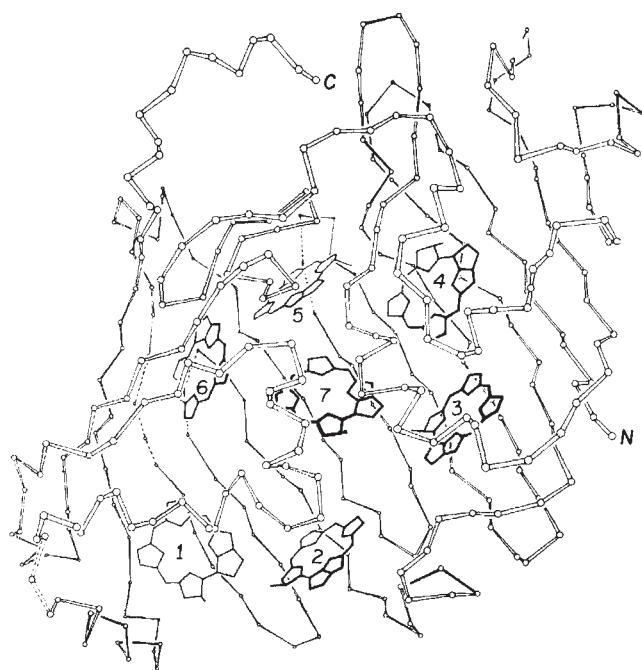


Fig. 1 Schematic diagram showing the arrangement of the polypeptide backbone and chlorophyll core of one subunit of the bacteriochlorophyll protein. Presumed α -carbon positions are indicated by circles. The connectivity of the polypeptide chain is in some instances uncertain (see text), and particularly ambiguous regions are indicated by broken lines. For clarity, the magnesium atoms, the chlorophyll ring substituents and the phytol chains, except for the first bond, are omitted. The direction of view is from the threefold axis, which is horizontal, towards the exterior of the molecule.

asymmetric carbon atoms in rings II, IV and V have been established^{17,18}. In addition the X-ray crystal structure of ethyl chlorophyll *a* (a *trans*-esterified derivative of chlorophyll *a* in which the phytol group is replaced by ethyl) has been determined¹⁹⁻²¹.

The seven Bchl *a* molecules are confined within an ellipsoid of axial dimensions: $45 \times 35 \times 15$ Å (Fig. 3). The average centre-centre nearest neighbour distance between porphine rings is 12 Å, which may be compared with 24 Å, the closest distance between chromophores in adjacent subunits. The orientations of the porphine rings do not conform to any repetitive pattern, although their planes do, to a first approximation, lie parallel with one another. This may be demonstrated by taking the mean of the direction cosines of the perpendiculars to each of the rings to define a plane which is most nearly parallel with the planes of the seven porphine rings. The angles made by the individual porphine planes with this calculated plane lie between 10° and 40°. In contrast, the azimuthal orientations of the near coplanar rings vary considerably. The normal to the calculated plane makes an angle of 62° with the threefold axis of the trimer and the centre of the aggregate in each subunit is at a distance of about 20 Å from the threefold axis. Thus, in the complete trimer the Bchl *a* aggregates of the individual subunits combine to form a triangular funnel about the threefold axis.

In each subunit the phytol chains lie close together, sandwiched between five of the porphine rings on one side and the other two rings and the β sheet wall of protein on the other. The hydrocarbon chains are in most cases in an extended conformation, although in one instance (Bchl 1) the chain is bent into a complete U-shaped loop. The phytol chains of chlorophylls 4, 5 and 6 lie parallel, forming a planar structure in close contact with the β -sheet wall of the protein. This hydrophobic core of phytol chains is completely buried in the protein, a thermodynamically favourable arrangement analogous to the interior of other globular proteins in which most of the hydrophobic residues are found buried out of contact with the solvent. A characteristic of β structure is the alternation of the directions of the amino acid side chains relative to the plane of the sheet. It seems likely that in this case the inwardly directed side chains are predominantly hydrophobic, forming close interactions with the phytol aggregate, whereas the residues directed towards the exterior are largely hydrophilic, forming favourable interactions with the solvent. The β sheet thus provides a suitable amphipathic layer, shielding the hydrophobic Bchl aggregate from the aqueous surroundings. Since the direction of the side chains alternates along the polypeptide chain, it will be interesting to see whether the sequence of these strands conforms to a pattern of alternating hydrophobic and hydrophilic residues.

The electron density map gives no evidence for chemical interactions between neighbouring chlorophyll molecules, either direct or through bridging water molecules, other than the hydrophobic associations which undoubtedly occur between the phytol chains. There is, however, good evidence for extensive interactions between the chlorophyll molecules and the surrounding protein shell. The presence of a fifth ligand to the Mg atom of each of the Bchl *a* molecules is indicated by electron density protruding from the centres of each of the porphine rings. In five cases this density extends to form a continuous bridge between the Mg atom and the polypeptide chain. Without knowledge of the amino acid sequence it is not possible at this stage to characterise these ligands. Peptide oxygens or amino acid side chains such as Asp, Glu and their amides, Ser, Thr, His or Met could interact with the Mg directly or by hydrogen bonding to a coordinated water molecule. In addition, the Bchl *a* ring substituents include a number of potential hydrogen bond acceptors such as the oxygen-

containing groups mentioned earlier. Most of these groups are close to the periphery of the aggregate and are sufficiently close to the protein electron density to be expected to form hydrogen bonds with suitable donors in the polypeptide chain. Thus, it seems that the Bchl *a* molecules are anchored to the protein through extensive hydrogen bonding and liganding to the Mg atom, in addition to hydrophobic interactions. The distribution and orientations of the chlorophyll molecules are therefore governed largely

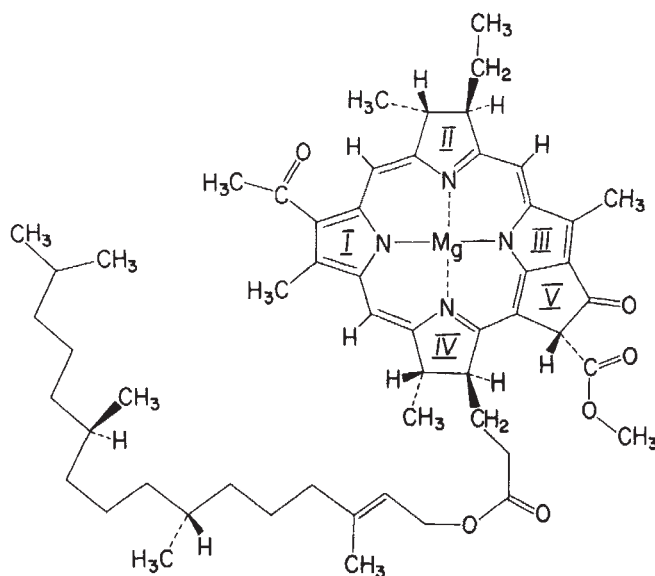


Fig. 2 Structure of bacteriochlorophyll *a*, indicating the absolute configurations of the asymmetric carbon atoms.

by specific interactions with the protein rather than with one another. The Bchl molecules do not occur in regular one- or two-dimensional arrays as has been observed in crystal structures of chlorophyll derivatives, and postulated as models for chlorophyll arrangement *in vivo*¹⁹⁻²².

Energy transfer

Fluorescence studies suggest that in the green photosynthetic bacteria this Bchl *a* protein functions as an intermediary in the transfer of excitation energy from the antenna pigment, chlorobium chlorophyll 660, to the photochemical reaction centre, P840. Mechanisms for energy transfer between the components of the photosynthetic apparatus of both bacteria and higher plants have recently been discussed by several authors²³⁻²⁵. Energy transfer between chromophores separated by relatively short distances (10–20 Å) may be described in terms of an exciton model²⁶⁻²⁸. For a given electronic transition, coulombic interactions between the transition dipole moments of neighbouring identical, or near identical, molecules in an aggregate give rise to a set of *N* discrete exciton energy levels, where *N* is the minimum number of interacting species. Splitting of the main 809-nm absorption band and the presence of a complex CD spectrum at liquid nitrogen temperatures have been observed for this Bchl *a* protein. These observations were interpreted in terms of an exciton interaction involving a minimum of five or possibly six Bchl *a* molecules with estimated nearest neighbour distances of 12–15 Å (ref. 6 and J. M. Olson, unpublished). The work described here shows that there are in fact seven Bchl *a* molecules separated by average nearest neighbour distances of 12 Å in each subunit of the trimer. The interactions between Bchl *a* molecules within each subunit

thus appear to be appropriately described in terms of an exciton model. The magnitudes of the exciton interactions are critically dependent upon the relative orientations of the transition dipoles and on the inverse cube of the distances between the chromophores. We have observed a high degree of non-uniformity in the azimuthal orientations of neighbouring Bchl molecules in this aggregate (Fig. 3). This observation is in good agreement with an interpretation of the complex low temperature absorption and CD spectra in terms of the exciton theory. Furthermore, since the positions and orientations of the Bchl *a* molecules are now known, it should be possible to calculate the expected exciton interactions, and hence estimate the numbers and magnitudes of the absorption splittings and components of the CD spectrum. Such calculations for model systems using experimentally determined dipole moments or calculated point monopoles have been described^{29,30}. It is hoped that this Bchl *a* protein

transfer would be required to occur over distances in the range of 20–50 Å (as for example between subunits of the Bchl-protein trimer) intermediary between the exciton theory and Forster's resonance transfer.

Chlorophyll proteins

The results described here represent the first structure determination of a chlorophyll-containing protein. In common with other globular proteins it contains the usual types of secondary structure such as α helices and β sheet. Unlike any other protein for which the X-ray crystal structure has been determined, however, it contains a large non-protein core as was originally proposed by J. M. Olson³⁵. Presumably both the protein and the Bchl are necessary to maintain the integrity of the complex. The molecule is also unusual in being trimeric, having a cyclic arrangement of subunits involving extensive heterologous interactions. The

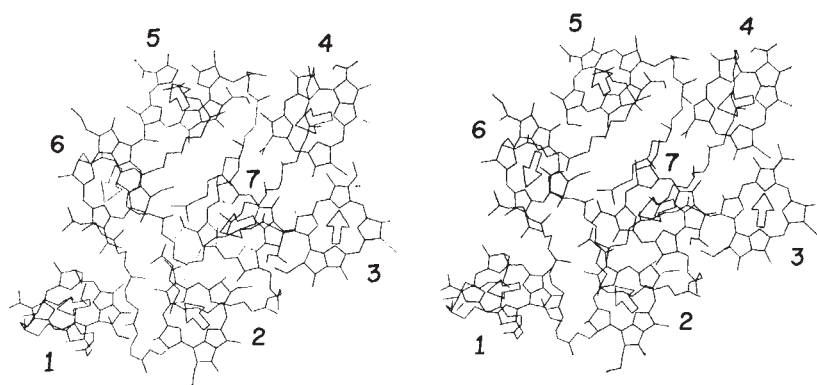


Fig. 3 Stereo diagram illustrating the chlorophyll arrangement within one subunit of the bacteriochlorophyll protein. The direction of view is approximately normal to the "average" chlorophyll plane (see text). The arrows, directed from ring III to ring I, are parallel to the Q_y transition moments of the respective bacteriochlorophylls. The magnesium atoms are omitted.

will provide a useful model for testing current theories of exciton interactions between chlorophyll molecules; so enabling more reliable structural evaluations to be made for other chlorophyll containing systems for which multi-component CD spectra have been observed^{31–32}.

In comparison with the Bchl protein described here, very little is known of the environments of the light-harvesting chlorobium chlorophyll 660 and the reaction centre, P840; and virtually nothing about the relative separation and spatial distributions of all three components³³. Any discussion of the mechanism of energy transfer to and from the Bchl *a* protein remains speculative in the absence of such information. At chromophore separations of 50–100 Å, energy transfer is thought to occur by resonance, which, unlike exciton transfer, has an inverse sixth power dependence on separation distances³⁴. If, however, the other chlorophyll components occur in proteins with molecular dimensions comparable with the Bchl *a* protein, and these proteins are closely associated in the membrane, then energy

functional significance of the trimeric nature of the molecule is obscure, though if the protein were extrinsically associated with the photosynthetic membrane it could indicate the presence of an intrinsic membrane component also possessing a threefold axis of symmetry.

Recent progress in the isolation and partial characterisation of a number of reaction centre and antenna chlorophyll proteins from photosynthetic membranes suggests that most, if not all, of the chlorophyll in both green plants and bacteria occurs in close association with relatively low molecular weight proteins^{36,37}. The Bchl *a* protein described here is somewhat atypical in that it is highly water-soluble, whereas most other chlorophyll-proteins require the use of detergents to facilitate their solubilisation from membranes. There is however no evidence at present to suggest that the organisation of chlorophyll in these hydrophobic proteins should be grossly different from the model we have described. All of the current models describing the state of chlorophyll *in vivo* place emphasis on either direct

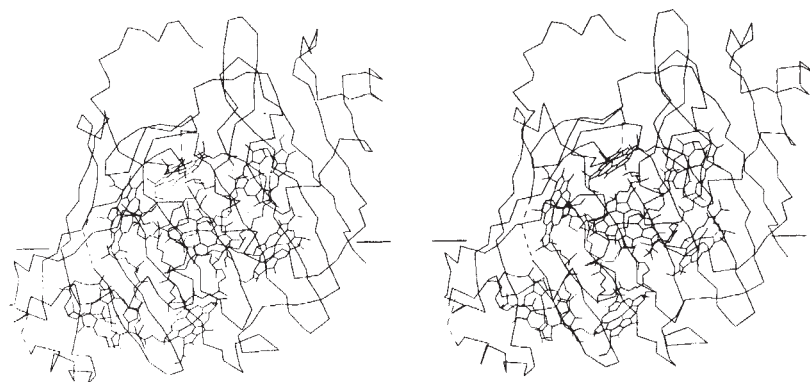


Fig. 4 Stereo drawing showing the arrangement of the chlorophyll core within the polypeptide envelope of the bacteriochlorophyll protein. The direction of view is the same as in Fig. 1, and the location of the threefold axis is indicated by the two horizontal lines.

interaction involving Mg liganding or hydrogen bonding via bridging water molecules as the major factors determining the relative orientations of the porphine rings^{19,38,39}. Our new findings for this particular Bchl-protein indicate that interactions between chlorophyll and protein including liganding to the magnesium atom, hydrogen bonding and hydrophobic interactions are of major importance in determining the arrangement of the chlorophyll molecules. Furthermore we suggest that these types of interaction could be of universal significance in determining the state of chlorophyll *in vivo*.

Our results may be summarised as follows: the three-dimensional structure of a chlorophyll-containing protein has been determined by X-ray crystallography and shown to consist of three identical subunits, each containing a core of seven bacteriochlorophylls enclosed within an envelope of protein. The bacteriochlorophyll molecules are confined within a flattened disk-shaped region with their porphine rings lying roughly parallel to the disk. In contrast to current models for chlorophyll arrangement *in vivo*, the chlorophyll packing is dominated by interactions between chlorophyll and protein rather than between chlorophyll and lipid or between adjacent chlorophyll molecules. Furthermore the chlorophylls are arranged in an irregular fashion rather than in strictly ordered one-dimensional or two-dimensional arrays. It is suggested that the arrangement of chlorophyll seen here, in close association with protein, typifies the usual arrangement of chlorophyll *in vivo*.

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Identification of two related pentapeptides from the brain with potent opiate agonist activity

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Enkephalin, a natural ligand for opiate receptors is composed of the pentapeptides H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH. The evidence is based on the determination of the amino acid sequence of natural enkephalin by the dansyl-Edman procedure and by mass spectrometry followed by synthesis and comparison of the natural and synthetic peptides.

TERENIUS and Wahlström^{1,2} and Hughes³ have described the existence of an endogenous substance in the brain

which acts as an agonist at opiate receptor sites. We later characterised this substance, termed enkephalin, as a low molecular weight peptide⁴. Other workers⁵ have also confirmed the presence of a substance in the brain that competes for opiate binding sites and this substance, although not completely characterised, seems similar to enkephalin. A further peptide with opiate agonist actions, larger and chemically dissimilar to enkephalin, has been discovered in the pituitary gland⁶. We have now found that enkephalin is composed of two pentapeptides which we have identified and synthesised.

Enkephalin was isolated from pig brains as previously

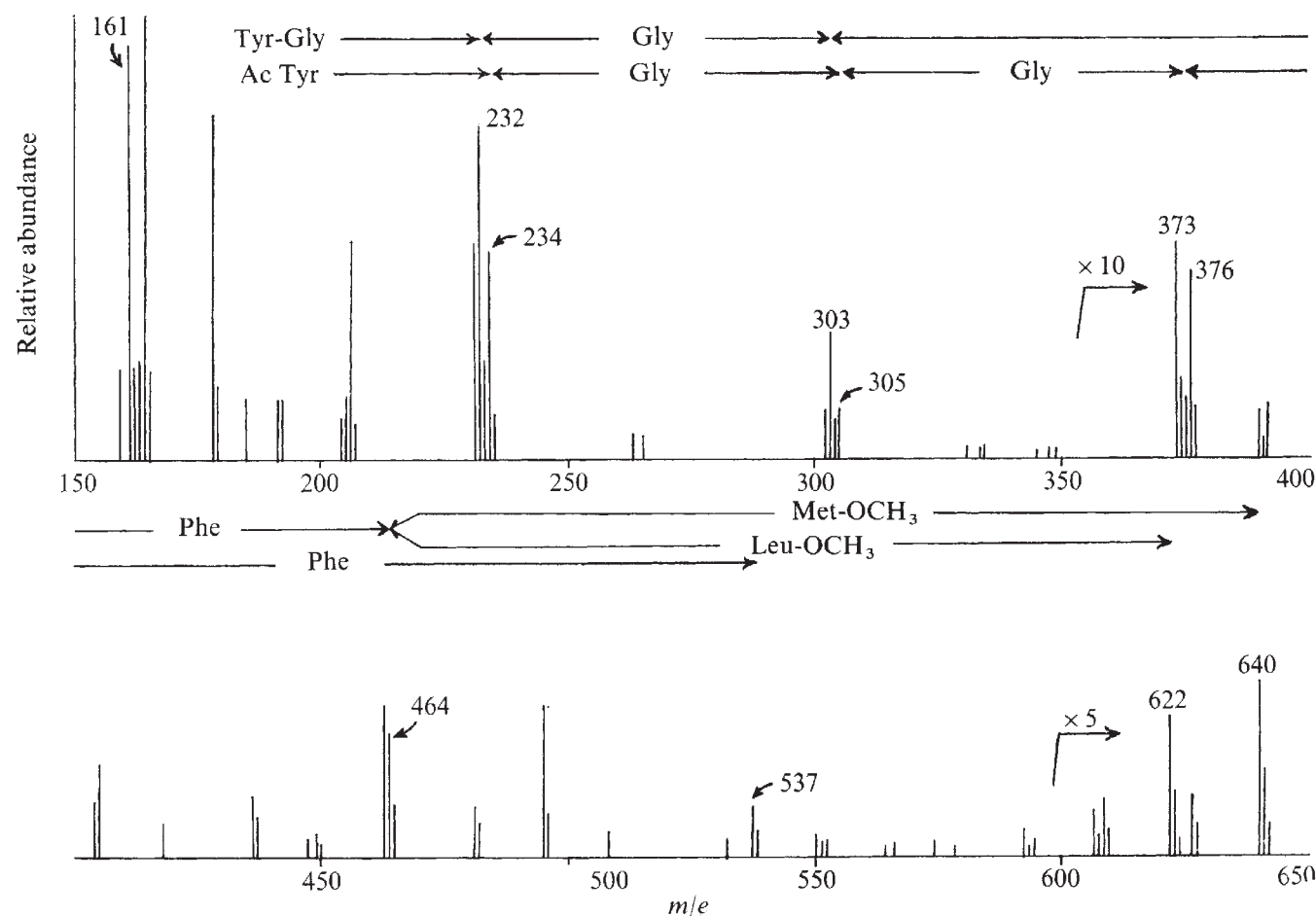


Fig. 1 Mass spectrum (above m/e 150) of the acetyl permethyl derivative of natural enkephalin. The assignment of the two pentapeptide sequences shown was based mainly on the recognition of an N-C cleavage pathway¹⁰; other modes of fragmentation, consistent with these structures, are present in the spectrum and will be described elsewhere. The spectrum also shows that enkephalin does not contain an unusual amino acid⁴.

described^{3,4}. The amino acid composition after hydrolysis with 5.5 N HCl at 110 °C for 20 h was: glycine 36.5, methionine 12.3, tyrosine 16.9, phenylalanine 22.5 and leucine 4.2 nmol. Tryptophan was not detected after hydrolysis with 3 N mercaptoethanesulphonic acid⁷ at 110 °C for 65 h.

The amino acid sequence was first investigated by sequential degradation using the dansyl-Edman procedure⁸. This indicated the sequence Tyr-Gly-Gly-Phe- for the first four residues. Two amino acids were tentatively assigned to the fifth position, one clearly corresponding to methionine sulphone and another corresponding to leucine but giving a weaker spot. No further dansyl derivatives were detected after position 5. The chain length of the peptide could not be definitely fixed either by amino acid analysis or by the dansyl-Edman procedure. Molecular weight determinations by electrophoretic mobility⁴ indicated a value of approximately 800.

The resulting uncertainties about the chain length and sequence assignment at position 5 were resolved by independent mass spectrometric analysis. The mass spectrum of the N-acetyl permethyl derivative⁹ of natural enkephalin is shown in Fig. 1. It is interpreted as arising mainly from a pentapeptide sequence H-Tyr-Gly-Gly-Phe-Met-OH where methionine is C terminal. This conclusion is substantiated by the results of deuterium labelling (m/e 640 shifts to m/e 658 on treatment with CD₃I (ref. 11)) and examination of enkephalin treated with cyanogen bromide (m/e 640 shifts to m/e 624). The presence of the m/e 622

signal is not entirely consistent with this interpretation. It seems to indicate the presence of H-Tyr-Gly-Gly-Phe-Leu-OH having a C-terminal leucine; alternatively it could be due to the loss of C₇H₇ from a putative molecular ion at m/e 713 for the methionine peptide.

The peptide sequences thus assigned to natural enkephalin from the mass spectra were synthesised by classical solution methods, the details of which will be published elsewhere. The mass spectrum of synthetic H-Tyr-Gly-Gly-Phe-Met-OH as the N-acetyl permethyl derivative has been found to be identical with that of natural enkephalin with the major exception that m/e 622 is absent in the synthetic material. On the other hand, the mass spectrum of the N-acetyl permethyl derivative of synthetic H-Tyr-Gly-Gly-Phe-Leu-OH differs from that of the natural enkephalin mainly by the absence of m/e 640 and the presence of m/e 622 as the most abundant high mass signals. The spectrum of the derivative of a mixture of the two synthetic peptides is identical with that of natural enkephalin. The mass spectrum of natural enkephalin is therefore interpreted as being the result of a mixture of the two pentapeptides H-Tyr-Gly-Gly-Phe-Met-OH (methionine-enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-OH (leucine-enkephalin). It is estimated that the enkephalin isolated by us has a ratio of methionine-enkephalin to leucine-enkephalin of 3 or 4 to 1. This conclusion is in agreement with the results of the amino acid analysis.

The electrophoretic mobilities of the natural enkephalin and of the synthetic methionine- and leucine-enkephalins

were determined at pH 2 and 2.5 kV on Whatman 3MM paper using 1-diaminonaphthalene-5-sulphonic acid as a neutral marker. A mobility of +0.61 relative to valine was found for natural enkephalin and its synthetic constituents. Mixtures of natural enkephalin with the synthetic peptides migrated as one spot.

The detailed pharmacology of the synthetic peptides will be presented in a later paper. Both methionine- and leucine-enkephalins have potent agonist activity at opiate receptor sites in that they produce a dose-related inhibition of electrically evoked contractions of the mouse vas deferens¹² and the guinea pig ileum¹³ (Fig. 2). These inhibitory effects could be completely antagonised by naloxone (Fig. 3). Methionine-enkephalin is twenty times more active than normorphine in the vas deferens and equipotent with normorphine in the guinea pig ileum (Fig. 2). Preliminary results on the inhibitory effects of methionine-enkephalin on the stereospecific binding of ³H-naloxone in Na⁺-free homogenates of guinea pig brain indicate that methionine-enkephalin is at least three times more potent than morphine. Leucine-enkephalin has half the potency of that of methionine-enkephalin in the vas deferens but in the guinea pig ileum only one-fifth of that of methionine-enkephalin.

Natural enkephalin has been found to be 5–10 times more active in the vas deferens than in the guinea pig ileum³, and the potency of natural enkephalin in the vas deferens, relative to normorphine, was found to be 15 to 1 in this study where the amount of enkephalin was

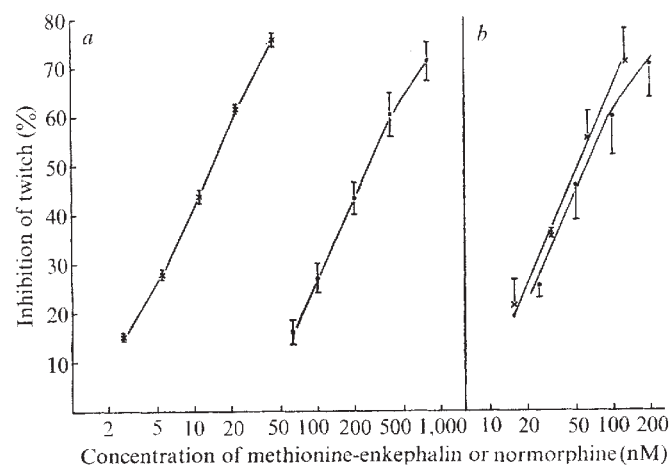


Fig. 2 Dose-response curves for normorphine (●) and synthetic methionine-enkephalin (×) in the mouse vas deferens (a) and guinea pig ileum (b). Potency ratios, enkephalin/normorphine: 21.5 ± 4.5 ($n = 4$) in the mouse vas deferens and 1.29 ± 0.35 ($n = 3$) in the guinea pig ileum.

determined by amino acid analysis. There is thus a very close agreement between the pharmacological activities of the natural and synthetic peptides.

In view of the correspondence between the natural and synthetic peptides in terms of their biological potencies, their mass spectra and electrophoretic mobilities, we conclude that our assigned structures for the two peptides comprising natural enkephalin are correct. At present, it cannot be decided whether the morphine-like peptides of Terenius and Wahlström¹ and Pasternak *et al.*⁵ are identical with the natural enkephalin characterised by us. It is particularly interesting that the methionine-enkephalin sequence is present as residues 61 to 65 in β -lipotropin isolated from pituitary glands of sheep¹⁴, pig¹⁵ and man¹⁶. Attention has already been drawn^{14–16} to structural relationships between β -lipotropin and β -MSH and ACTH. It is tempting to speculate that a similar relationship may exist between β -lipotropin and the peptides with opiate agonist activity obtained by Goldstein and colleagues from

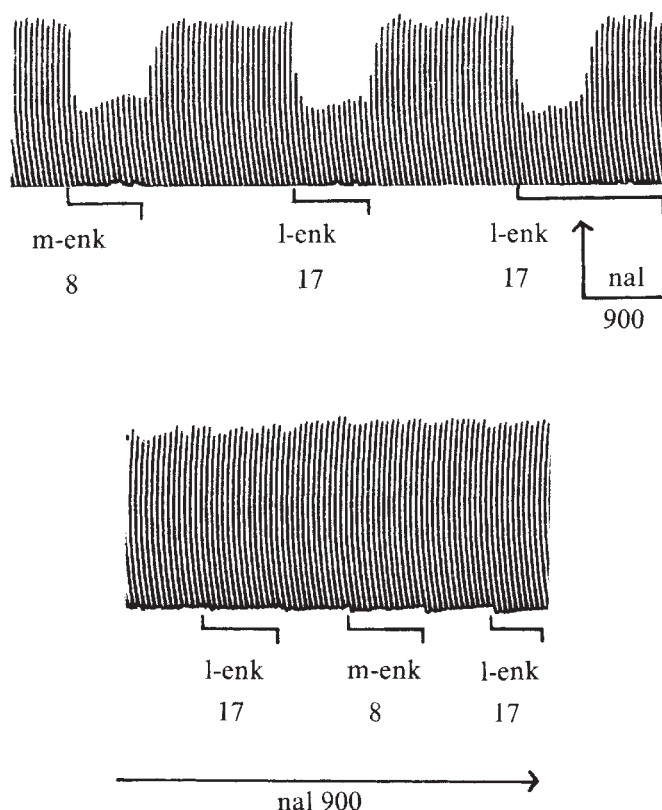


Fig. 3 The depressant effects of methionine-enkephalin (m-enk) and leucine-enkephalin (l-enk) on the electrically induced contractions of the mouse vas deferens, and the antagonist effect of naloxone (nal) which was present throughout the lower tracing. The numbers give concentrations in nM. The concentration of naloxone (900 nM) was chosen to give complete reversal of the inhibition; 275 nM caused an 80% reversal.

bovine pituitary¹⁷ and crude ACTH¹⁸ and that β -lipotropin may be a common precursor for these peptides and methionine-enkephalin.

The discovery in the brain of two endogenous pentapeptides with potent opiate agonist activity raises a number of pertinent questions which cannot be adequately dealt with in this paper. It will now be possible, however, to test the hypothesis¹⁹ that these peptides act as neurotransmitters or neuromodulators at synaptic junctions.

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Mechanism of catalysis of hydrocarbon reactions by platinum surfaces

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Atomic height steps and kinks in steps have been identified on platinum surfaces as distinct catalytic sites where C-C, C-H and H-H bond scissions occur during the reactions of hydrocarbons. The active catalyst surface is covered with a carbonaceous overlayer whose properties affect both the reaction rates and the production distribution. A model for the catalysis of hydrocarbons on platinum surfaces is proposed which incorporates the effects of the low coordination number active sites and the atomic surface structure.

We have studied the correlation between the activity of platinum crystal surfaces carrying out a variety of catalytic reactions of hydrocarbons, and their atomic surface structure and surface composition. We report here the identification of distinct atomic sites on the platinum surface where H-H, C-H and C-C bond breaking predominantly occur. The hydrogen-deuterium exchange reaction¹ provided information about the H-H bond breaking, whereas the C-H and C-C bond breaking activity was determined by monitoring simultaneously the rates of dehydrogenation and hydrogenolytic ring opening of cyclohexane to produce benzene and *n*-hexane, respectively². Experimental evidence also indicates that the catalytically active surface is covered with a carbonaceous overlayer which forms as a result of partial dehydrogenation of hydrocarbons^{2,3}. The overlayer is either ordered or disordered, dependent on reactant and clean platinum surface structure, the ordering influencing both the product distribution and the rate of surface reactions.

The surface structures of the clean platinum single crystals and of adsorbed molecules were studied by low-energy electron diffraction, and the surface composition by Auger electron spectroscopy⁴. The rates and product distributions in the various chemical reactions were measured by a quadrupole mass spectrometer at low pressures³ and a gas chromatograph at high pressures⁵. The surface area of the single crystal catalyst samples was about 1.0 cm². Reaction rates on the catalyst surfaces as low as 10⁻¹⁵ mol cm⁻² s⁻¹ were readily detectable.

Active sites on platinum surfaces

Three types of platinum surface, which are distinguishable by their reactivities, are shown schematically in Fig. 1. The low Miller Index Pt(111) surface (Fig. 1a) has a very low density of surface imperfections, <10¹² cm⁻², as compared with the number of surface atoms, 1.5 × 10¹⁵ atoms cm⁻². The high Miller Index Pt(557) surface (Fig. 1b) is characterised by a stable surface structure with 18% of the surface atoms in monatomic height steps⁶. The Pt(679) surface (Fig. 1c) must have, in addition to high step density, a high density of kinks in the step or atoms of lower coordination since the (679) plane does not lie between two close packed planes as does the (557) plane [(111) and (001)]⁴. Thermal stability of high Miller Index surfaces has been systematically investigated, and their relative stability as a function of crystal orientation, and relative activities in various hydrocarbon reactions have been determined⁷. The different surface sites are distinguishable by their coordination number, that is, their number of nearest

neighbours, ranging from kink atoms with only 6, to atoms in (111) orientation terraces with 9.

Molecular beam scattering indicates the reaction probability (number of reactive collisions per total collisions with surface) for H-D exchange increases by four orders of magnitude from <10⁻⁵ on the Pt(111) surface to ~0.1 on a highly stepped platinum surface, Pt(355)¹. The presence of a high concentration of atomic steps was necessary to detect the formation of HD on single scattering at low pressures, and the amount of HD formed was proportional to the step density.

We have also found that the dehydrogenation of cyclohexane and cyclohexene to benzene occurs at an appreciable rate only on stepped platinum surfaces^{2,8}. Figure 2 demonstrates this by showing the dependence of the rate on step density for the latter reaction. The rate of cyclohexane dehydrogenation to benzene is constant as long as there are steps (tested as low as 7% steps) on the catalyst surface, but is almost an order of magnitude lower on the Pt(111) surface, as seen in Fig. 3. Thus atomic steps seem to be the preferred surface sites for breaking H-H and C-H bonds.

The relative rates of dehydrogenation and hydrogenolysis for cyclohexane can best be monitored by the ratio of benzene to *n*-hexane in the reaction products. As shown in Fig. 3, the rate of benzene production is independent of step and kink density, while *n*-hexane production increases slowly with step density and rapidly with kink density. The rate of *n*-hexane production per kink site is determined by the slope of the line in Fig. 3b, which gives 1 × 10⁻²⁰ mol s⁻¹ per kink atom. This is almost an order of magnitude higher than the slope in Fig. 3a which corresponds to 1.2 × 10⁻¹⁹ mol s⁻¹ per step atom. The observed low hydrogenolysis activity on the type of stepped surfaces represented in Fig. 1b may be caused by thermally generated kinks in the steps⁹. Since the formation of *n*-hexane and other hydrogenolysis products must be the result of C-C bond scissions, it seems that kinks are very effective in breaking C-C bonds as well as C-H and H-H bonds. Thus we have been able to identify two active sites of lower coordination number on platinum surfaces, steps with C-H and H-H and kinks in steps with C-C, C-H and H-H bond breaking activities.

The carbonaceous overlayer

During the experiments, the catalyst surfaces were always covered with a carbonaceous overlayer²⁻⁴. The coverage was almost independent of pressure ranging from 10⁻⁴ to 10⁵ Pa (unpublished results of this laboratory) but varied markedly with temperature and the molecular weight of the saturated hydrocarbon reactant molecules². The higher the temperature and the reactant molecular weight, the higher the coverage, reaching monolayer amounts for cyclohexane at 725 K or *n*-heptane at 575 K. Unsaturated hydrocarbons, such as ethylene or benzene, form complete monolayers at all temperatures and pressures, and double layers in certain conditions^{7,10}, with the reactant adsorbed on the overlayer. The overlayer consists of partially dehydrogenated hydrocarbon species²⁻⁴ formed from the dissociation of the reactants. This carbonaceous overlayer may be ordered or disordered depending on the platinum surface structure, the nature of the reactant

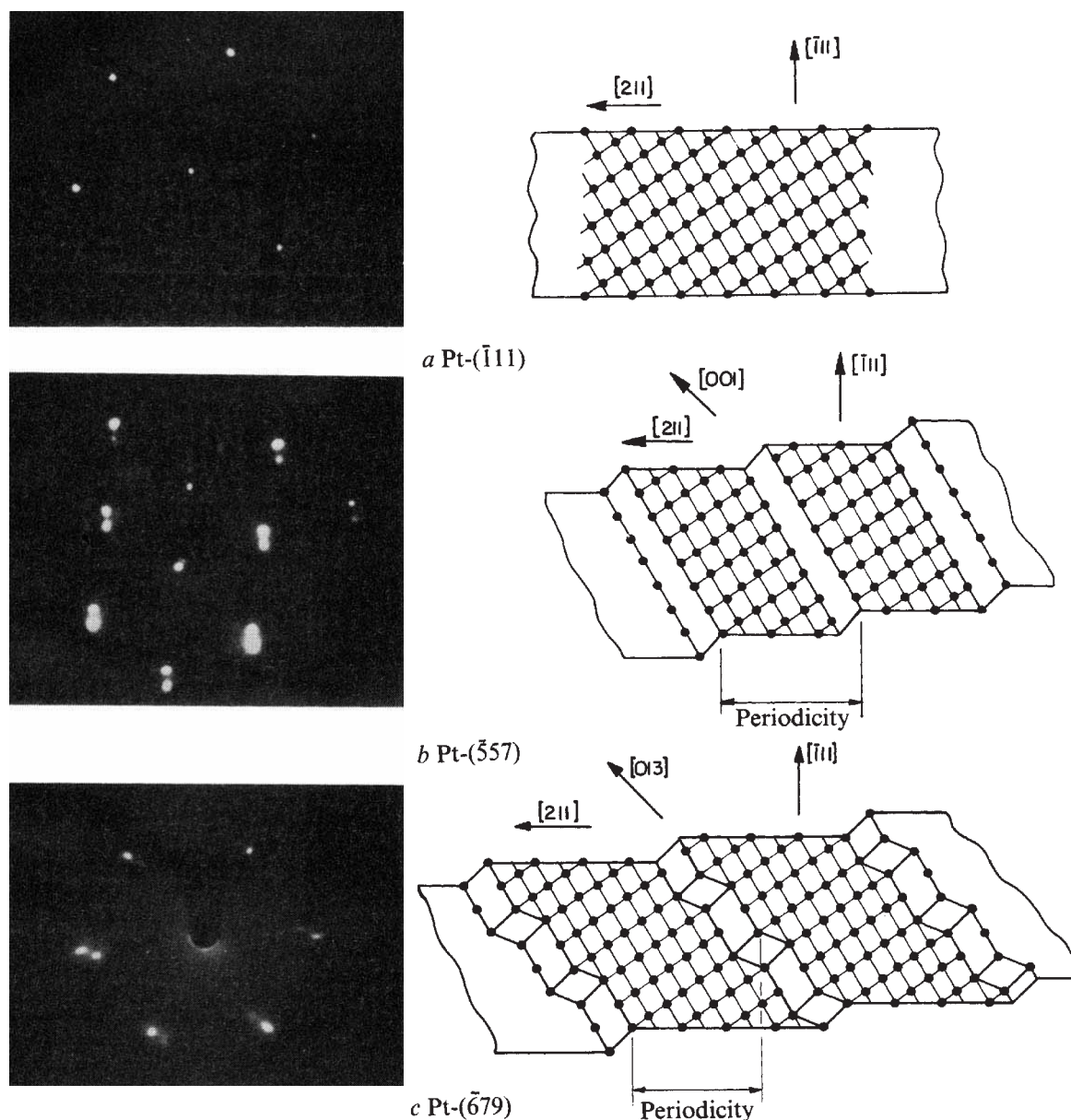


Fig. 1 Low energy electron diffraction patterns and schematic representations of the mean surface configurations of three representative platinum single crystal catalyst surfaces. *a*, Pt($\bar{1}11$) surface containing $< 10^{12}$ defects cm^{-2} ; *b*, Pt($\bar{5}57$) surface containing 2.5×10^{14} step atoms cm^{-2} , with an average spacing between steps of 6 atoms; *c*, Pt($\bar{6}79$) surface containing 2.3×10^{14} step atoms cm^{-2} and 7×10^{13} kink atoms cm^{-2} with an average spacing between steps of 7 atoms and between kinks of 3 atoms.

and the hydrogen–hydrocarbon ratio used in the experiment. Several reactions are very sensitive to the presence of ordering on the overlayer². Cyclohexene conversion to benzene is poisoned unless the overlayer is ordered, and *n*-heptane to toluene conversion occurs only in the presence of an ordered overlayer³. Other reactions like the hydrogenolysis of cyclohexane occur readily even in the presence of a disordered overlayer.

Model of the active platinum catalyst

As a result of these experimental observations we would like to propose an atomic model of the platinum surface active in the catalysis of hydrocarbon reactions (see Fig. 4). The surface is heterogeneous both in atomic structure and in composition. The steps and kinks are the active sites where C–H and H–H and C–C, C–H and H–H bond breaking occur, respectively⁴.

Although steps and kink sites exhibit higher binding energy for both hydrogen and hydrocarbons, the greatly increased

rate of dissociation of hydrogen at steps¹ may keep these sites active. Even though these sites are areas of high catalytic activity, the product desorption probably occurs from areas covered with the carbonaceous overlayer. Diffusion on, and desorption from, the areas covered by the carbonaceous overlayer is likely since the binding energy of products should be lower there. The structure of the carbonaceous overlayer is important in more complex reactions like dehydrocyclisation of *n*-heptane to toluene and in reactions where rapid deactivation of the catalyst occurs. Thus, two types of structure sensitivity are to be distinguished, step structure sensitivity and overlayer structure sensitivity.

Since much of the structure sensitivity of the catalytic reactions reported in the literature has been deduced from the dependence (or independence) of the reaction rate on the mean particle size of dispersed catalyst particles^{11,12}, caution should be exercised in interpreting the mean particle-size dependence of the reaction rate in terms of our model. Some of the structural features may vary in proportion to changes of particle

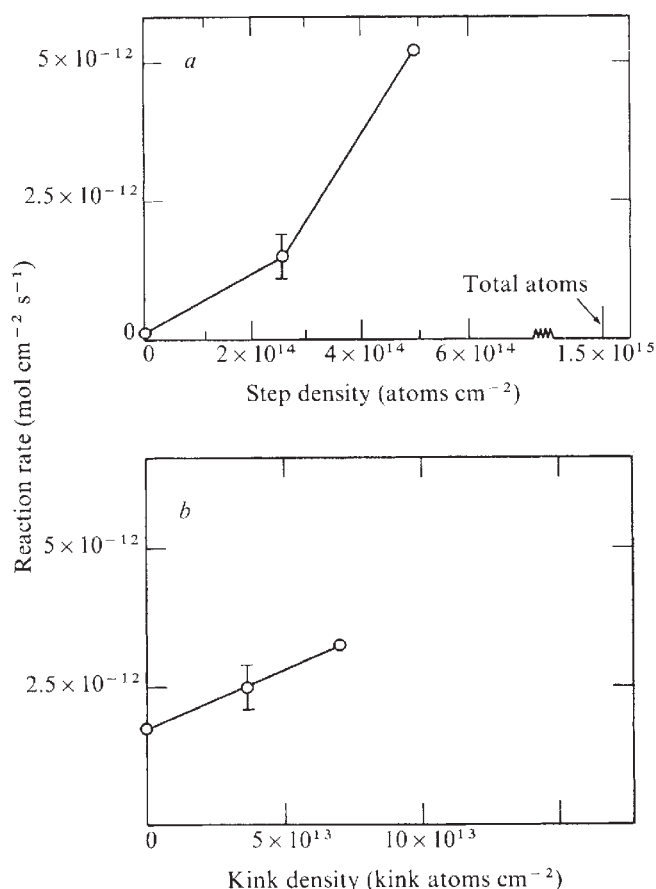


Fig. 2 Initial rate of cyclohexene dehydrogenation to benzene on platinum single crystal catalysts with *a*, increasing step density and *b*, increasing kink density at a constant step density of 2.5×10^{14} step atoms cm⁻². The reaction conditions are 5×10^{-6} Pa of cyclohexene, 1.0×10^{-4} Pa of hydrogen and 423 K catalyst temperature. Hydrogen-hydrocarbon ratio 20 : 1

size (perhaps step density) while other structural features (step-kink site ratio, overlayer structure) may not. Our experimental observations support the active site concept originated by Taylor¹³ for the importance of the heterogeneity of atomic surface structure in catalysis. Our model also accommodates the structure sensitivity-insensitivity reaction classification proposed by Boudart¹².

It should be noted that in our studies of platinum catalytic activity, the effect of the porous material used to support the highly dispersed metal particles in actual catalysts has not been taken into account. The support may influence the concentration and structure of the atomic steps and the carbonaceous overlayer. Most of the catalytic reactions that are attributed to the platinum in supported platinum catalysts¹⁴ have, however, been reproduced on the single crystal surfaces in the absence of the support. Thus, we do not expect that our model of the platinum catalyst is markedly changed when any effects of the support are taken into account.

Although many of our reaction studies on single crystal surfaces are carried out at both low^{2,3} (10^{-4} Pa) and at high⁵ pressures (10^5 Pa) the rate determining steps in the mechanisms of surface reactions might be expected to change with the more than eight orders of magnitude change in pressure. Whether the structural features that control the platinum catalysis of hydrocarbon reaction will be less influential at high pressure than at low pressure will have to be determined by future experiments.

The causes for the unique bond breaking activity of low coordination number sites (steps, kinks) on surfaces has been the topic of theoretical concern¹⁵⁻¹⁸. The extent to which the variation of chemical behaviour at surface irregularities is

the property of transition metals other than platinum is yet to be determined. Although palladium stepped surfaces exhibit higher heats of hydrogen chemisorption than the low Miller Index surfaces¹⁹, gold, a non-transition metal, exhibits oxygen and hydrocarbon chemisorption properties that are identical on both stepped and low Miller Index surfaces²⁰.

The model of the catalytically active platinum surface also explains the effect of selective poisoning that influences the product distribution in hydrocarbon reactions so markedly. An impurity, a second component or a poison like sulphur, present in small concentrations, will block first the kink sites that are of the highest binding energy on the heterogeneous surface, drastically reducing the C-C bond breaking activity (hydrogenolysis rate), and only moderately affecting the catalytic reactions where only C-H and H-H bond breaking is required (dehydrogenation or hydrogenation). This way selectivity in product distribution may be achieved.

It should be pointed out that the two important structural features that characterise the heterogeneous platinum catalyst surface—atomic steps and carbonaceous overlayers—also emphasise the relationship of heterogeneous catalysis to homo-

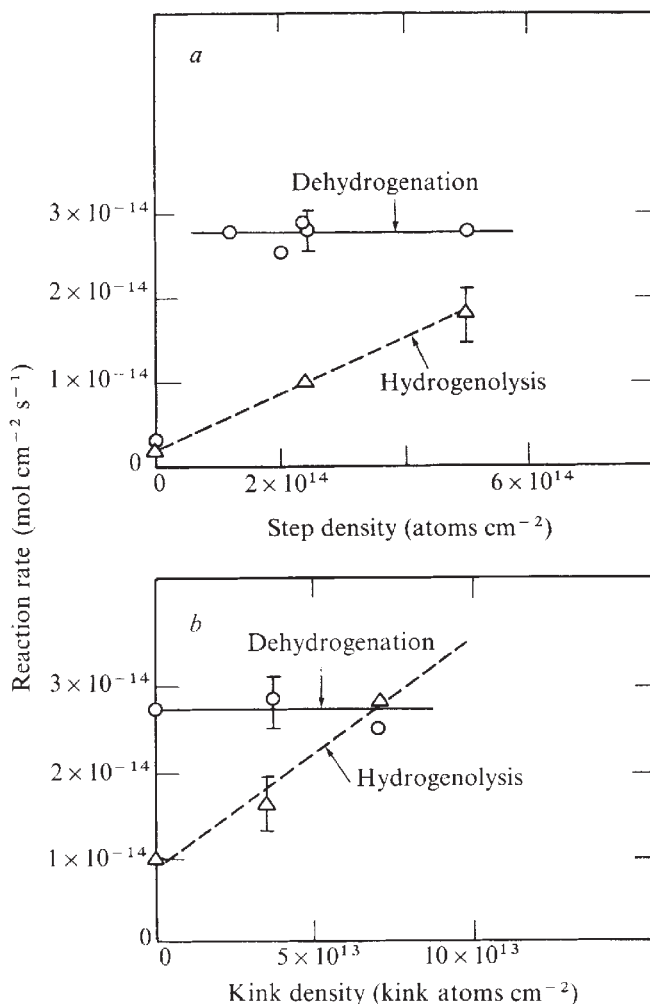


Fig. 3 Initial steady state rates of cyclohexene dehydrogenation to benzene (○) and hydrogenolysis to *n*-hexane (△) on platinum single crystal catalysts with *a*, increasing step density and *b*, increasing kink density at constant step density of 2.4×10^{14} step atoms cm⁻². The reaction conditions are 5×10^{-6} Pa of cyclohexene, 1.0×10^{-4} Pa of hydrogen and 423 K catalyst temperature. The rate of dehydrogenation is constant at 2.8×10^{-14} mol cm⁻² s⁻¹ with steps present on the surface but is less (0.3×10^{-14} mol cm⁻² s⁻¹) on the Pt(111) surface (0.0 step density). The rates of hydrogenolysis per surface site at the slope of the lines representing hydrogenolysis are 1×10^{-20} mol s⁻¹ per kink atom and 1.2×10^{-19} mol s⁻¹ per step atom. Hydrogen-hydrocarbon ratio 20 : 1.

geneous and enzyme catalysis. The catalytic activity or bond breaking ability of clusters of atoms along steps or near kinks on the heterogeneous metal surface may be correlated with

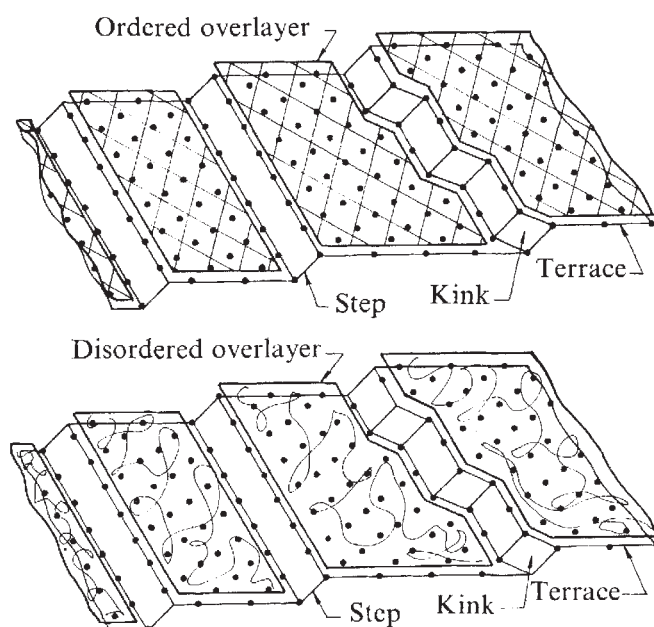


Fig. 4 Model of active platinum catalyst surface with a full carbonaceous overlayer showing exposed catalytic sites.

the reactivity of homogeneous systems with a single metal atom or metal clusters surrounded by ligands. The metal-ligand catalysts will break and form H-H and C-H bonds as do the metallic clusters, but generally lack the ability to break C-C bonds which is possessed by the highly uncoordinated kink atoms on metallic clusters. The presence of ordered carbonaceous overlayers which apparently are necessary to perform the complex rearrangement of large molecules, for example the dehydrocyclisation of *n*-heptane to toluene, or to prevent catalyst deactivation may be correlated with the structure sensitivity and specificity so important in complex enzyme reactions.

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letters to nature

A spherical accretion model of Cyg X-1

THE binary X-ray source Cyg X-1 is widely believed to be a black hole, and most current models for explaining its X-ray spectrum¹ assume an accretion disk. The reason for requiring a disk, rather than a spherical accretion flow, is that earlier investigations of the properties of spherically accreting black holes seemed to indicate that, for the mass transfer rates required to give the observed luminosities of 10^{37} - 10^{38} erg s⁻¹, the cooling time of the gas would be so short compared to the free-fall time that the gas would arrive essentially cold at the Schwarzschild radius, and there being no solid surface to transform the infall kinetic energy into thermal energy, very little of this would be radiated away²⁻⁴.

In fact, spherical accretion on to a black hole can convert gravitational energy into radiation with an efficiency comparable with that of disk systems⁵. Earlier results, indicating that if

$$L = \eta \dot{M} [GM/r_s] \quad (1)$$

(where $r_s = 2GM/c^2$ is the Schwarzschild radius) then $\eta \ll 1$, neglected to take account of the spherical inflow being necessarily subject to magnetohydrodynamic and plasma turbulence, and that this provides a viscous dissipation mechanism which heats the gas far more efficiently than the $p dV$ work. That the spherical accretion flow must be turbulent follows, if nothing else, from the fact that the large scale shear $\sigma \sim (GM/r^3)^{1/2}$ leads to a growth of whatever initial frozen-in magnetic field

is present², up to values $H^2/8\pi \gtrsim GM\rho/r$ (Since $H^2 \propto r^{-4}$ but $GM\rho/r \propto r^{-5/2}$). This near-equipartition value cannot be exceeded, because the magnetic energy can only be generated at the expense of the gravitational energy; reconnection of field lines must occur, leading to plasma instabilities and to turbulence being continually regenerated. Although equipartition magnetic fields were considered in previous spherical calculations, the heating arising from the turbulent viscous dissipation, and the Joule heating arising from the field line reconnection were not included. That such dissipative heating must occur, however, is necessary for the same reason as in disk models, namely, that inflow could not proceed without it: the excess magnetic energy must be dissipated before the magnetic pressure tries to halt the collapse, and the angular momentum associated with random transverse turbulent motions has to be carried away (for example by collision between blobs) to allow the gas to move inwards.

We may estimate the magnitude of the heat input rate per unit volume, Γ , from the condition that the turbulent energy content of a volume bounded by r and $r/2$ must be dissipated in the turbulent time scale ($\sim$ free-fall time), or that the magnetic energy content in the same volume be dissipated in the Alfvén time scale. If both of these energy forms are near equipartition with the gravitational energy (which makes the Alfvén time approximately equal to the free-fall time as well) then this condition is

$$\Gamma r^3 t_{ff} \sim \bar{\rho} v_t^2 r^3 \sim \left(\frac{H^2}{8\pi} \right) r^3 \sim \frac{\beta}{4} \left(\frac{GM\rho}{r/2} \right) r^3 \quad (2)$$

where v_t is turbulent velocity, t_{ff} is free-fall time and $\beta \lesssim 1$. The heat input rate is therefore

$$\Gamma \sim 4 \times 10^{32} r^{-4} n_a \beta \left(\frac{M}{10 M_\odot} \right)^3 \left(\frac{v}{2 \times 10^8 \text{ cm s}^{-1}} \right)^{-3} \text{ erg cm}^{-3} \text{ s}^{-1} \quad (3)$$

where M is the mass of the central compact object, n_a is the baryon number density at the accretion radius $r_a = GM/v^2$, $v = (v_w^2 + v_s^2)^{1/2}$ with v_w = wind velocity and v_s = sound velocity at r_a , and the free-fall law for the density was assumed, $\rho \propto r^{-3/2}$. The inclusion of this dissipative heating has the effect of making the efficiency factor η of equation (1) close to unity for densities such that the cooling times are shorter than the free-fall times, quite unlike the result of previous calculations. The fact that the turbulent field is continuously excited by field line reconnection and plasma instabilities, and that this magnetic-turbulent energy must be dissipated, ensures that gravitational energy is converted into heat at all radii down to the Schwarzschild radius without the need to invoke a solid surface to halt the flow. This mechanism should therefore have a role in neutron star, or white dwarf accretion as well. Most of the luminosity comes from the regions nearest to the Schwarzschild radius r_s , since

$$L(r) \sim 4r^3 \Gamma(r) \propto n_a M^3 r^{-1} \quad (4)$$

and provided that $t_{cool} \lesssim t_{ff}$ at least near to r_s an efficiency η close to unity is ensured by the fact that $H^2/8\pi$, or ρv_t^2 must build up to equipartition as a consequence of flux-freezing. Thus, spherical accretion flows can be just as efficient as disk systems, for accretion on to a black hole.

The main observational properties of Cyg X-1 can be reproduced by such a spherically accreting black hole, without going into a very detailed calculation. We take as an example $M = 10 M_\odot$, and adopt the accretion radius $r_a = 3.3 \times 10^{10}$ cm, with a wind velocity $v_w \sim 10^3$ km s $^{-1}$ and a sound velocity v_s appropriate to a temperature $T \sim 3 \times 10^7$ K. To have a luminosity $L \sim 10^{37}$ erg s $^{-1}$, from equation (1) we require a density at the accretion radius $n_a \geq 10^{10} \eta^{-1} \text{ cm}^{-3}$, or $M \simeq 4\pi r_a^2 v \rho_a \gtrsim 5 \times 10^{16} \eta^{-1} \text{ g s}^{-1}$. In the absence of any Compton scattering, the X-ray spectrum is due to bremsstrahlung, and the spectrum is $L_\nu \propto r^3 \epsilon_\nu(r) \propto v^{-1/2}$, where $\epsilon_\nu(r) \propto n(r)^2 T(r)^{-1/2}$ is the differential emissivity per unit volume. Since Cyg X-1 has a spectrum that, at least part of the time, is steeper than this, it seems necessary to invoke Compton scattering to reproduce the spectrum. In the disk calculation of Shapiro *et al.*⁶, it is shown that, for an electron temperature $T_e \sim 10^9$ K, and for the Compton scattering parameter $y = (4kT_e/m_e c^2) \tau_{es} \simeq 1$ (where τ_{es} is the Thomson optical depth) then a solution of the photon transport equation in phase space (Kompaneets equation) is able to reproduce the spectrum $L_\nu \sim \nu^{-1}$ as required by observations, provided that a copious supply of soft photons [energy 50 eV $\lesssim h\nu_\lambda \lesssim 5$ keV] is present. Since, however, the cyclotron mechanism, which is the likeliest source of soft photons, has a ground harmonic frequency far below 50 eV, they have to invoke either recapture of radiation from regions further out, or postulate arbitrary soft photon sources in the gas. We can adapt this Compton scattering calculation to the spherical case, by choosing similar parameters, without in fact having to invoke an arbitrary soft photon source, or recapture. The cyclotron mechanism produces photons in the higher harmonics as well, and in fact most photons are not at the ground harmonic, but rather at the harmonic ν_m at which the cyclotron opacity $\tau_{\nu, cy}$ drops below unity, and this is usually a very high harmonic. Below ν_m , the cyclotron mechanism gives a Rayleigh-Jeans spectrum $L_\nu \propto \nu^2$, and above ν_m it drops abruptly. Choosing $n_a = 2 \times 10^{12} \text{ cm}^{-3}$, we have $n = 4 \times 10^{17} \text{ cm}^{-3}$ and $\tau_{es} \simeq 2.5$ at a radius $r = 2 r_s = 10^7$ cm. The equipartition average magnetic field is $\langle H \rangle = 3.5 \times 10^7$ and the cyclotron frequency $\langle \nu_H \rangle = 10^{14}$ Hz at that radius, and using

the expression⁷ for the quasi-continuous cyclotron opacity coefficient $\kappa_\nu = (4\pi^2 v_F^2 / c v_H) A \exp(-av/v_H)$, one finds that $\nu_m \simeq 4 \times 10^{15}$ Hz at an assumed temperature $T \simeq 10^9$ K. These values are based on the mean values $\langle H \rangle$ and $\langle v_H \rangle$, but in fact the field line reconnection and dissipation should lead to inhomogeneities, so that the blobs doing most of the emission and absorption of photons should have values of H and v_H higher than the average. It is very difficult to estimate the mean square fluctuations, but it seems quite likely that $\nu_m \sim 1-2 \times 10^{16}$ Hz, $h\nu_m \gtrsim 40-80$ eV as required for input photons in the Compton scattering process. The spectrum above ν_m would then be a power law in the energy, with index ~ -1 up to an energy $h\nu \sim kT \sim 10^2$ keV and dropping off above that. The total luminosity from Compton cyclotron radiation is $L \sim \text{few} \times 10^{37}$ erg s $^{-1}$, indicating that the assumed temperature of $T \simeq 10^9$ K is approximately the right temperature for radiating away the thermal energy produced by the dissipation-reconnection mechanism of equation (3) with $10^{-1} \lesssim \beta \lesssim 1$. Since most of the radiation arises from within a few Schwarzschild radii, the millisecond time variability of Cyg X-1 is explained by the usual light-travel time arguments, the total luminosity corresponds to what is observed, and the overall features of the spectrum up to hard X-rays, are as satisfactory as in the latest improved disk models. The intensity and spectrum changes (for example the 1971 downward transition, with a harder spectrum) may be explained by changes in the accretion rate. A lowering by a factor ~ 4 of the mass transfer rate would proportionally reduce the total luminosity and the Thomson optical depth. The less efficient Compton cooling leads to a higher temperature near the Schwarzschild radius, and to a harder spectrum with a flatter power law exponent.

A spherical model may be necessary in Cyg X-1 if the mass transfer in the binary system is not fed by Roche lobe overflow, but rather by a wind^{8,10,11}. Even if there was enough angular momentum to form a disk, or even a marginal, unstable disk⁹, the thin disk approximation used would not be valid in the hard X-ray emitting regions, since height $\sim$ radius. In such circumstances, spherical geometry seems a closer representation of reality than a disk, and furthermore does not require spin flipping of a specialised nature, extraneous photon sources or uncoupled electron and proton temperatures (although these last two could be accommodated). A spherical model therefore seems to afford a certain economy of assumptions.

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B0924 + 30 an unusual radio galaxy

THE radio source B0924 + 30 (ref. 1) has been studied as part of a systematic survey of radio galaxies of relatively low ($< 10^{41}$ erg s $^{-1}$) radio luminosity. The radio source is identified with a 14 mag elliptical galaxy with a redshift $z = 0.0266$ (refs 2 and 3). A 12-h observation of this radio source was made with the Westerbork Synthesis Radio Telescope⁴ at 1.4 GHz in September 1974, and Fig. 1 is a contour plot of the brightness distribution obtained. The resolution is 23'' in right ascension and 46'' in

Table 1 Positions and flux densities for the components of B0924+30

External component	<i>a</i>	<i>b</i>	<i>c</i>
Position RA	09h 25m 50.0±0.1s	09h 24m 24.8±0.1s	09h 23m 33.9±0.1s
dec.	30° 20' 45±2"	30° 07' 18±2"	29° 58' 33±3"
Angular distance from galaxy	14'	8'	22'
Flux density at 1.4 GHz (10^{-29} W m ⁻² Hz ⁻¹ mJy)	51±3	54±3	62±3
Flux density at 0.6 GHz (mJy)	84±13	76±12	71±14
Spectral index	-0.6±0.2	-0.4±0.2	-0.2±0.2
Component diameter	< 10"	< 10"	< 10"
Linear distance from galaxy (kpc)	322	187	508
Luminosity (W Hz ⁻¹ sr ⁻¹)	10 ^{22.5}	10 ^{21.5}	10 ^{21.6}
Position angle of vector between nucleus and component	55±15°	54.5°	53°
		53°	52°

declination. The r.m.s. noise level in the map is 0.5 mJy. For a single 12-h observation the map is distorted by elliptical grating rings of semiaxis $10' \times 20'$. The grating rings from many of the small diameter sources have been removed, but the distortion from the extended part of the radio galaxy remains and is the cause of the low level negative regions to the north and south of the galaxy. No correction for the primary beam attenuation, which reaches a factor of 2 at the edge of the map, has been applied. The position of the nucleus of the elliptical galaxy is RA = 09 h 24 min 55.1 s, dec. = 30°12'15", and is indicated by the + in Fig. 1.

The extended source is in itself interesting—it shows clearly an S-shaped structure with respect to the galaxy, already noticed in less striking form in some other radio galaxies, notably in Cyg A⁵ and 3C98 (ref. 7).

Even more intriguing are the three point-like sources (*a*, *b*, and *c*) in Fig. 1, which have almost equal intensity and lie on a line passing through the nucleus of the elliptical galaxy at the same position angle as the extended components. In addition to the 1.4 GHz map we have a set of 7 short (7 min) observations made with the Westerbork Synthesis Radio Telescope at 0.6 GHz. Although the map made from these observations has

much higher noise (r.m.s. ~ 4 mJy) than the 1.4 GHz map, it is adequate to estimate the flux densities of the components. The three point-like sources have spectra which are somewhat flatter than average. Table 1 gives the positions and flux densities of all components, and the linear dimensions and luminosity assuming a Hubble constant of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$.

Precise alignment between the compact components and the nucleus of the parent galaxy has already been seen in other cases (for example, Cyg A⁵ and DA240 (ref. 6)), but two features are unusual in this case:

First, the compact components lie well outside the region of extended emission. Although rarer, this may also have been observed in some other radio galaxies, such as 3C61.1 (ref. 7). Here, however, the radio emission extends all the way to the compact components. Second, there are three, rather than two, compact objects involved, and this has not been seen previously.

Of course the most important question to ask is whether this unusually striking alignment necessarily implies a physical association. A definite answer to this question cannot depend on the application of statistics after the event, but must await either the discovery of further similar configurations, or direct evidence for a connection such as an emission bridge.

Fig. 1 Contour map of the vicinity of the radio source B0924+30 at 1.4 GHz. The contours are at equal intervals in steps of 1.0 mJy per synthesised beam area (0.6 K). Negative contours are broken and the zero contour is not shown. The + indicates the position of the 14 mag galaxy.

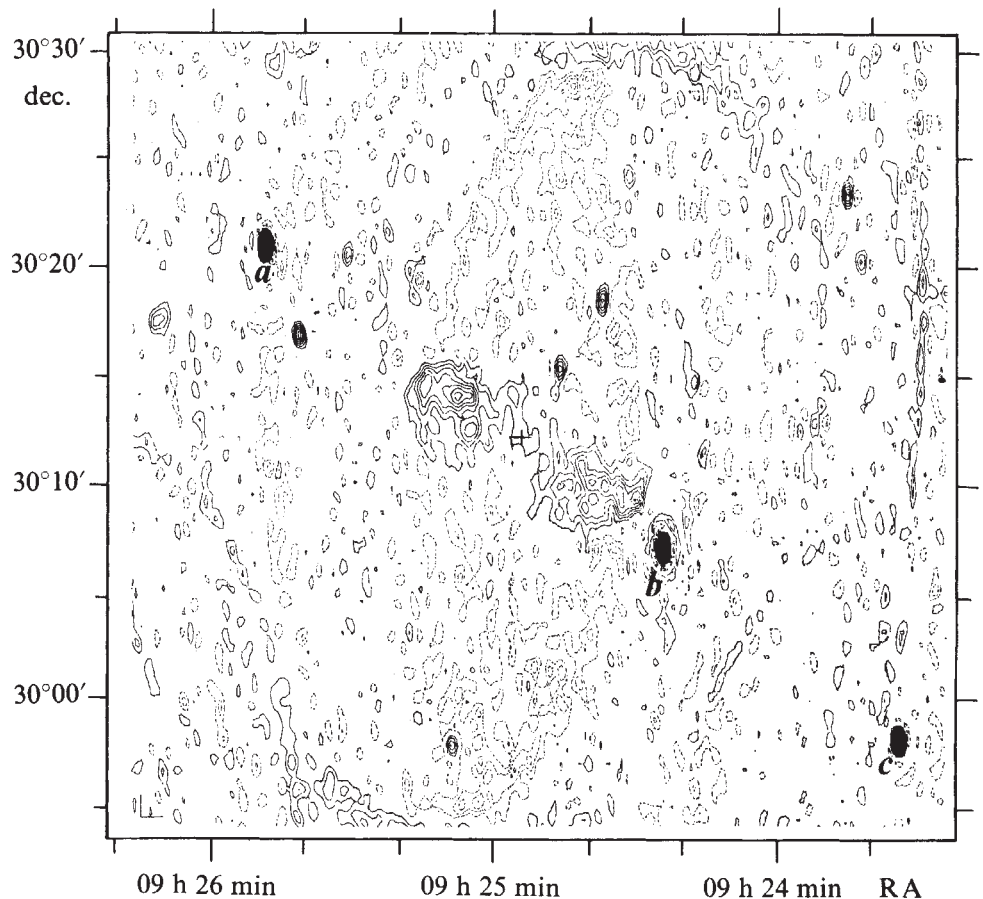


Table 2 Probability of chance alignment

Probability	Event
1.3	One source ≥ 50 mJy in primary beam area.
0.022	A second source lying within $\pm 1.5^\circ$ of the line from the first source to the galaxy.
0.022	A third source within $\pm 1.5^\circ$ of the same line.
0.17	Probability that the line through the three sources agrees with the position angle of the extended source to within $\pm 15^\circ$.
0.16	Probability that all three sources have equal intensity within a factor of 1.4 (observed ratio is 1.2).
Combining these we obtain a final probability of 1×10^{-5} .	

No similar configurations have been previously seen, but there has been a strong observational selection against such objects. There is selection against low luminosity objects in complete radio source surveys—the components in our case are rather weak compared with the noise level in most maps, and they are sufficiently far away from the parent galaxy that either the identification would not be noticed, or the area mapped would be too small to contain them. We know of ~ 10 observations of low luminosity radio galaxies in which such a configuration would have been seen, but was not.

We have searched for any possible bridge connection between these components by smoothing the 1.4 GHz map to lower resolution. A possible connection can be seen between component *b* and the tip of the extended source but this is not significantly ($\sim 1.5\sigma$) above the noise. Further observations are planned at 0.6 GHz to improve the sensitivity to weak extended structure and at 5.0 GHz to improve the resolution on the compact components.

Since we have no other case of this type, and no strong evidence for connections, it is necessary to comment on the estimated probability of occurrence of such an alignment. The density of radio sources at this flux level at 1.4 GHz is $1.3 \times 10^4 \text{ sr}^{-1}$ (estimated from the observations of Katgert *et al.*⁸); hence, it is not unlikely that some sources at this level will be seen in the 10^{-4} sr area of our primary beam. We give in Table 2 the steps in a calculation of the probability of an alignment by chance so that the reader can judge for himself the merit of each step.

To make this estimate as meaningful as possible we have used only probability calculations with some *a priori* justification, namely: First, the alignments seen between compact components and the nucleus of the galaxy in Cyg A, DA240 are $\leq 1.5^\circ$. Second, other compact components related to the parent galaxy lie within the angle subtended by the extended component. Third, the median intensity ratio for normal double radio sources is 1.4 (refs 9 and 10).

The positions of the three aligned components have been searched on the prints of the Palomar Sky Survey, and in one case, source *b*, there is a stellar image with ultraviolet excess near the plate limit. The position of this object is: RA = 09 h 24 min 24.8 $\pm$ 0.1 s, dec. = $30^\circ 07' 18.0 \pm 0.8''$, which is within $2''$ of the radio position. This is within the errors in the radio position and would normally be considered as a rather certain QSO identification.

Clearly if this association of the three compact radio components with the extended radio source and its parent galaxy is a physical association it will have important implications for the theory of formation of double radio sources. There are three rather than the more usual two components; the compact components lie well outside of the region of extended emission; they are aligned with the inner part of the S-shaped extended source rather than the outer part. On the other hand, without further examples of the same type it would be premature to exclude the possibility that one or more of these components are unrelated field sources.

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Selection of quasars by variability

THE first step in gleaning cosmological information from quasars is to determine their spatial distribution and luminosity function, based on well defined, complete samples^{1,2}. This idealised situation applies to a great extent in the case of radio quasars. For the majority of quasars, which are radio quiet, however, existing samples based on ultraviolet excess are not only incomplete, but are dominated by colour selection effects³. The relative importance of these effects depends on redshift, and, in particular the detection of high redshift quasars is extremely difficult⁴; consequently other methods of selection of radio-quiet quasars and BL Lac-type objects have been suggested^{5,6}. Here, one such method based on optical variability is considered, with particular regard to a sample of 13 objects observed by van den Bergh *et al.* (BHP)⁶.

Variability has long been known to be an important characteristic of quasars⁷. Its use as a major selection criterion is, however, jeopardised not only by contamination from other variable objects, but also by uncertainties about the nature of the subgroup of quasars which are selected.

There are three main types of variable objects which will contaminate a 'variability selected' sample. These are luminous variable stars (mainly RR Lyrae), low luminosity late M-type flare stars and N-type galaxies⁸. Provided samples are obtained in directions well out of the galactic plane, the number of RR Lyrae and other luminous variable stars should be very low. The number of late M-type flare stars and N galaxies are, however, virtually independent of direction, and also increase with fainter limiting magnitude. The contaminating effects of these objects can, to a certain extent, be mitigated by the introduction of further weak colour selection. In particular late M-type flare stars would have extreme red colours, and thus could be removed from a sample, although some quasars might be missed in this way. A closer study of the light curves of all the objects in a sample would result in the RR Lyrae and other luminous variable stars being recognised as such, although this could select against quasars whose variation resembled these objects.

A more fundamental problem is that of the nature of the quasars selected and their relationship to quasars in general. The probability of a quasar being in a sample depends on the form of its luminosity curve with time. For example, periodic variation would be detected more easily than flare behaviour. Also, small variations would not be detected, especially in objects whose brightness was close to the limiting magnitude of a sample. The final, and possibly most serious effect, arises if variability is correlated with any other quasar parameters (for example, redshift), in which case redshift-dependent selection effects of an unknown nature would be operating. Note that any correlation found⁹ between variability and high

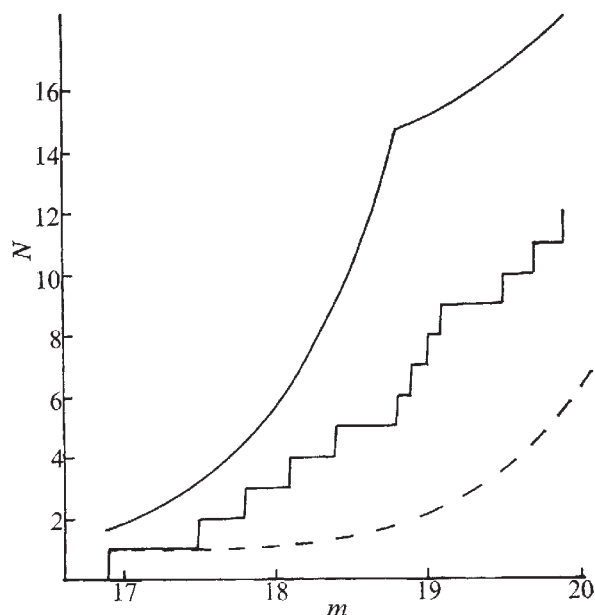


Fig. 1 The total number of objects in the BHP sample as a function of apparent magnitude (step function). The lower curve is the expected number of contaminating objects, and the upper curve includes the number of radio-quiet quasars, based on ultraviolet excess selection methods.

intrinsic luminosity is probably a selection effect. Since the number of quasars increases rapidly with decreasing apparent brightness, a variable quasar in a sample is much more likely to be a much fainter quasar which has brightened its apparent magnitude (and absolute magnitude) therefore being an overestimate. It would thus seem that variable quasars were, on average, of higher luminosity than non-variable quasars, giving rise to the correlation. This effect can be seen in Weistrop's sample¹⁰, where quasar candidates which were initially selected by colour, and then studied for variability, tended to decrease in magnitude after the initial selection.

In our variability selected sample, the numbers of contaminants were estimated as follows:

Luminous variable stars. The calculations were based on Field I of the Palomar-Groningen variable star survey (PG1)¹¹. The survey is centred on galactic coordinates $l=0^\circ$, $b=29^\circ$, and has a limiting magnitude of ~ 19 mag, although the sample is less complete towards fainter magnitudes. Variable stars are detected by blinking plate pairs, and allowance for incompleteness can be made using the statistics of the number of stars detected on 1, 2, ... 10 plate pairs. The BHP field is centred on $l=120^\circ$, $b=-20^\circ$ and a modified selection criterion is employed, in which objects are only deemed variable if they appear on at least two independent plate pairs out of the total of 21. This enables objects to be selected to a much fainter limiting magnitude, while at the same time avoiding contamination of the sample by spurious variables caused by grain statistics. The number of variables in PG1 is estimated to be 130, of which 42 are detected on at least two out of ten plate pairs. This number should not increase at fainter magnitudes, since all the variables within the Galaxy should be bright enough to be detectable. An extrapolation of these numbers for the 21 plate pairs of the BHP field, and a reduction by a factor ~ 10 to take into account the relative directions of PG1 and the BHP field results in 0.9 luminous variables, or approximately half of the variables being detected in the 6.2 square degrees of the BHP field¹². Although the reduction by a factor of 10 is very uncertain, the number of luminous variables expected should not be more than 2.0.

Late M-type flare stars. Since most stars of type M5 and later exhibit flares of more than 0.5 mag variation¹³, and five out of the 100 nearest stars are of this type¹⁴, the number of these stars per square degree down to a limiting magnitude m_V is

$$4.1 \times 10^{-7} \times 10^{0.2(3m_V - 27.6)} \quad (1)$$

where an absolute magnitude of $M_V \sim 14$ mag has been assumed¹⁴. About half of the variables would be detected, so that down to $m_V = 19.2$ mag (the approximate limiting magnitude of the BHP sample) ~ 1.2 late M-type flare stars would be detected. This figure is a lower limit since the number of flare stars with $M_V > 16$ is unknown. Also, since these stars would be within about 100 pc of the solar neighbourhood, they will be distributed fairly randomly over the sky.

N-type galaxies. According to W. Sargent (private communication), there are about 1.0 N galaxies per square degree down to the limit of the Palomar Sky Survey prints. If it is assumed that $\sim 25\%$ are detected, then 1.5 N-type galaxies are expected in the BHP field.

Quasars. We use the number-magnitude relation based on quasars found by the ultraviolet excess method of selection¹⁵

$$\log N = -0.27 + 0.6(m_V - 17.8); m_V < 18.8^m \quad (2)$$

where N is the number of quasars per square degree and a mean value of $m_B - m_V = 0.2$ mag has been employed. N is assumed constant for magnitudes fainter than $m_B = 19$ mag ($m_V = 18.8$ mag)

Figure 1 shows the total number of objects in the BHP sample as a function of apparent magnitude (step function). The lower curve is the number of contaminating objects and the upper curve includes the number of quasars according to equation (2). The BHP sample therefore contains approximately 65% quasars (1.2 per square degree) and provides about half the number of quasars generated by ultraviolet excess selection methods, but without the accompanying redshift-dependent selection effects. Variability selection therefore can provide comparable numbers of radio quiet quasars and BL Lac-type objects as other methods of selection, provided that the limiting magnitude is within the range 19 to 20 mag and, as in the BHP sample, directed away from the galactic plane. The number of quasars detected can be increased still further either by using a greater number of plate pairs, or by extending the limiting magnitude so that variable objects of up to 20 mag can be detected on one plate pair.

It should be noted that two of the objects in the BHP sample (denoted j and k) are separated by only $22''$. They may be variable stars or, if quasars, similar to the interesting quasar pair 1548+115 (ref. 16).

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Photosynthesis of organic compounds in the atmosphere of Jupiter

THE substances responsible for the red-brown colours of the atmosphere of Jupiter are the source of much debate. Initially it was suggested that the coloured materials are HCN oligomers and polymeric organic nitriles which are

formed during electrical storms on Jupiter^{1,2}. This model has been criticised on two grounds: first it seems unlikely that there could be sufficient lightning on Jupiter to account for the intense red-brown coloration and second, the electrical storms could only take place in the lower atmosphere of Jupiter, where condensation of water vapour takes place, and therefore there would have to be some means of transporting the compounds above the NH₃ ice clouds³. Another postulate is that the colour comes from elemental sulphur and polysulphides formed by the photolysis of H₂S (ref. 4). Organic compounds may also be produced when H₂S is photolysed in the presence of CH₄ and the other hydrocarbons present in the Jovian atmosphere^{5,6}. Since neither H₂S nor NH₄SH has been detected in the upper atmosphere of Jupiter it is necessary to postulate either that the rate of H₂S photolysis is much faster than its rate of transport to the upper atmosphere, or that sunlight reaches the lower atmosphere through gaps in the ammonia ice clouds and the sulphur and polysulphides so formed are then transported to the upper atmosphere. A third postulate suggests that both organic compounds and polysulphides may be formed by a bombardment of the atmosphere with high energy protons and electrons⁷.

Table 1 Reactants and products from the photolysis of CH₄, NH₃, H₂, and He mixtures*

Reactants	Pressure (mm Hg)	mol	Reactant consumed or photoproduct (mol)
NH ₃	30	2.87 × 10 ⁻³	1.8 × 10 ⁻³ †
CH ₄	95	9.15 × 10 ⁻³	1.5 × 10 ⁻³ †
H ₂	474	45.33 × 10 ⁻³	~4 × 10 ⁻³ ‡§
He	60	5.75 × 10 ⁻³	
N ₂			0.83 × 10 ⁻³ †
HCN			7 × 10 ⁻⁷ †
N ₂ H ₄			1 × 10 ⁻⁸ †
C ₂ , C ₃ hydrocarbons			

* The results given are an average of three runs in which the gaseous mixture was photolysed in a 1.8-l flask for 1 h using the low pressure mercury lamp, and the extent of NH₃ decomposition was measured spectrophotometrically using a 10-cm cell. CH₄, N₂ and the C₂ and C₃ hydrocarbons were detected and analysed by mass spectrometry⁹. He was used as the standard in the quantitative mass spectral analysis. HCN was detected and determined colorimetrically^{10,11} after the reaction products were dissolved in H₂O. Both methods indicated the same yield. N₂H₄ was analysed by specific colour test¹². Care was taken to use N₂H₄-free NH₃. All reactants were shown to be pure by mass spectral analysis.

† Amount consumed.

‡ Amount formed.

§ An approximate yield. Wide variation in yield was observed.

|| No quantitative analyses were performed.

We reported that the NH₃ in the upper atmosphere of Jupiter is subject to rapid photodecomposition⁸. Here we observe that an efficient conversion of CH₄ to hydrocarbons and HCN takes place when NH₃ is photolysed in the presence of CH₄, H₂, and He using a 184.9-nm light source (see Table 1). Of particular significance is the observation that photolysis of 1 molar equivalent of NH₃ results in the loss of 0.84 molar equivalent of CH₄. The CH₄ undoubtedly reacts with hot hydrogen atoms formed by the photolysis of NH₃ to NH₂ and H. The 92% yield of N₂ demonstrates that 8% of the NH₃ is not converted to N₂. Presumably this NH₃ is converted to organic amines and nitrile derivatives such as HCN. The high yield of H₂, approximately 30% greater than the stoichiometric amount expected on NH₃ photolysis, is consistent with hot hydrogen atom reactions¹³⁻¹⁵. This increased yield of H₂ reflects the H₂ formed by the condensation of CH₄ to higher molecular weight hydrocarbons.

These results demonstrate that NH₃ photolysis results in the very efficient conversion of CH₄ to more complex organic compounds in the upper atmosphere of Jupiter. This pathway for hydrocarbon synthesis explains how ethane

and acetylene are formed in the upper atmosphere of Jupiter¹⁶ since C₂ hydrocarbons were detected in our experiments. From the results of our photochemical studies we predict the presence of HCN, NH₂NH₂ and higher hydrocarbons in the Jovian atmosphere above the NH₃ clouds. Further condensation of these photoproducts may yield organic compounds responsible for the red-brown coloration in the upper Jovian atmosphere. Since this postulate requires neither the synthesis of the chromophoric compounds in the lower Jovian atmosphere, nor their transfer to the upper atmosphere, it is not subject to the criticisms discussed already for models requiring the use of electrical discharges or H₂S photolysis.

Although these results are primarily of significance to photochemical reactions of the Jovian planets and some of their satellites, they may also be applicable to the primitive Earth. Our previous results suggest that any atmospheric NH₃ would have been rapidly photodestroyed in the 1-10 Myr period after a significant atmosphere developed^{17,18}. NH₃ photolysis could have resulted in the efficient formation of organic molecules if this primitive atmosphere contained significant amounts of CH₄ or CO along with the NH₃ (ref. 19).

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Variation in time of mercury anomalies at the Mid-Atlantic Ridge

MERCURY concentrations vary markedly from place to place in the world ocean^{1,2}, we report here variations with time in bottom waters over the Mid-Atlantic Ridge at the FAMOUS area. Three sets of water samples from the axial rift valley have been examined since our original report of anomalous Hg in that region³. In 17 months Hg concentrations varied from 1,100 to 70 ng l⁻¹ (Table 1; Fig. 1).

Samples have been analysed using the cold vapour atomic absorption method (without modification)². Water was handled and stored according to the techniques recommended by Bothner and Robertson⁴. Sampling containers, procedures and personnel were provided by us on the first, third and fourth occasions. On the second, we obtained samples of water from polyethylene containers of known history allowing us to accept the data as a lower limit for mercury in the water at that time. Every sample was taken within 1.5 miles of 36°49'25"N, 33°15'40"W; bottom depths averaged 2,530 m. Samples from up to 230 m above

Table 1 Sample data

Date	Hg (ng l ⁻¹)*	Number of samples	Distance above bottom (m)	Sampling
October 1973	1,080 (1,420-870)	14	10	M. M. Jones from N.R.L. towed fish Niskin bottle rosette, USNS MIZAR (T-AGOR-11)
July 1974	250 (362-195)	6	1-ALVIN 0-180 KNORR profile	Dr H. Holland, Harvard University, WHOI, RN KNORR, ALVIN
October 1974	67 (83-58)	7	0-230 profile	This work; Niskin bottle hydrocast, USNS MIZAR (T-AGOR-11)
March 1975	78 (161-12)	5	0-175	R. Nekritz, D. Greenwalt, Niskin bottle hydrocast USNS BARTLETT (T-AGOR-13); Dr P. Taylor, SSOB, J. Ackerman, NavOceano

*Median value (range in parentheses).

the bottom were used since vertical profiles showed no identifiable trends.

Our results show clearly that large Hg anomalies can exist at certain times whereas normal oceanic concentrations prevail at others. We have not determined the source or sink for these anomalies. One attractive hypothesis is that there are intermittent hydrothermal injections from active ridge spreading centres^{3,5}. Local temperature anomalies of up to 0.1 °C have been reported for the FAMOUS site⁶, similar in magnitude and character to those presented by Scott *et al.*⁷, for another Mid-Atlantic Ridge site at 26°N, 45°W. Whatever the source, no information is yet available concerning the rate of injection and dissipation. Therefore, we cannot distinguish between a single injection with slow removal rate, or multiple injections with more rapid removal. Mercury has been found in close combination with the particulate phases in natural waters⁸, so removal is probably attributable to advection and/or the sedimentation of particles over periods of days to months, though mercury enrichment of nearby sediments has not been established as it has for sediments of the East Pacific Rise⁹.

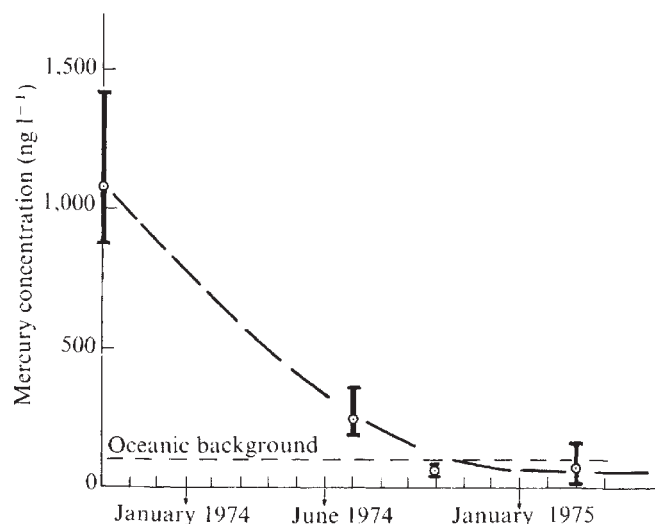


Fig. 1 Variation in time of the mercury content of bottom water around the Mid-Atlantic Ridge: ---, median value; bars indicate range.

The October 1973 samples, which showed drastically increased mercury concentrations, did not exhibit increased Fe, Mn or F concentrations³. These elements are often enriched in areas of tectonic activity. The 'normal' amount of these elements in seawater is from 10 to 1,000 times higher than that of Hg; so considerably larger inputs would be needed to show similar perturbations. Furthermore, new inputs of Fe and Mn to seawater are removed to the sediments very rapidly and very close to the injection point¹⁰.

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Origin and ancient history of gneisses older than 2,800 Myr in Lewisian Complex

THIS work records new field observations (to be discussed in detail elsewhere) which suggest that the pre-2,800 Myr history of the Lewisian Complex in the type localities of Scourie and Laxford, north-western Sutherland, was closely comparable with that of Godthåb, western Greenland. In western Greenland Precambrian rocks have been grouped into older (Amitsoq) and younger (Nuk) gneisses¹, separated in time by the emplacement of metamorphosed mafic-ultramafic Ameralik dykes. Supracrustal rocks and layered cumultic intrusions, probably younger than the Amitsoq gneisses but older than the Nuk gneisses are also present and have all been varying affected by high-grade amphibolite or granulite facies metamorphism. Although the Lewisian Complex contains gneisses which, together with supracrustal and layered cumultic igneous rocks, have suffered the widespread granulite facies metamorphism dated at about 2,800 Myr BP and can be compared with those of western Greenland^{1,2}, equivalents of the Amitsoq and Nuk gneisses have previously not been distinguished.

All the Lewisian outcrops discussed here are cut by dolerite dykes or their metamorphosed equivalents, belonging to the early Proterozoic Scourie Dyke Swarm. Between Badcall and Scourie, where the dykes retain igneous minerals, the gneisses are at granulite facies and yield dates of about 2,800 Myr (ref. 3).

In that region the oldest identified rocks are gneisses, in which layered basic igneous intrusions have been emplaced. The gneisses also contain medium-grained, homogeneous,

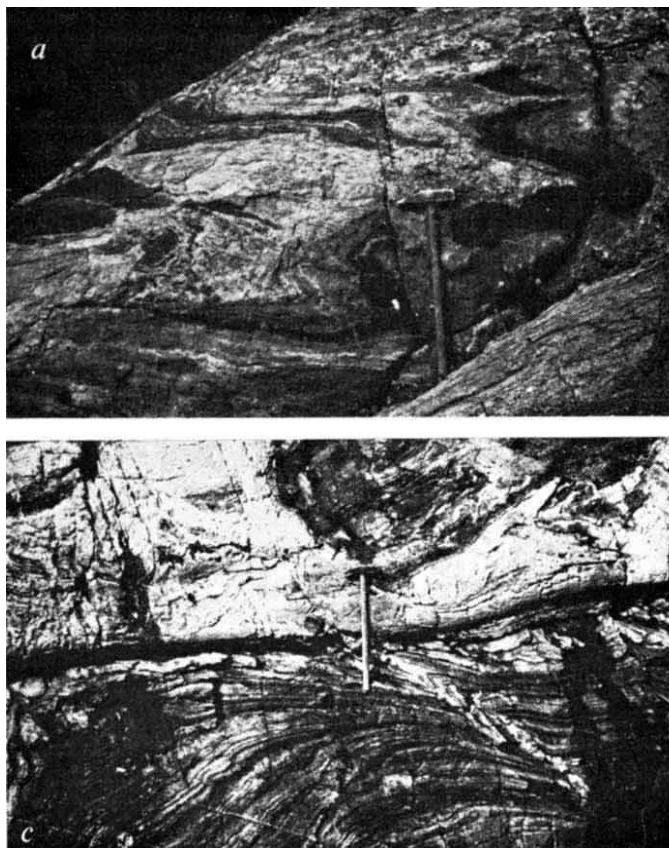


Fig. 1 *a*, Folded, discordant, ultrabasic dyke cutting old banded gneiss. On coast about 1 km south of Scourie. *b*, Younger intrusive gneiss in garnet-leucogabbro. Badcall, south of Scourie. *c*, Old banded gneiss, intruded by homogeneous younger gneiss (light coloured) which encloses rafts of garnet-leucogabbro. Badcall.

ultramafic-mafic layers, which are often highly deformed, broken up and migmatized. Where later deformation and migmatization have had least effect, the layers retain discordant relationships to irregular banded gneiss and have a dyke-like habit. Where these metadykes are least deformed it is clear that the bulk of the oldest recognised rocks are leucocratic medium to coarse grained, quartzofeldspathic gneisses with which are intercalated dark pyroxene-bearing bands up to 1 m thick. Large feldspars up to 2 cm in diameter occur in the darker gneiss lithologies. Ultramafic layers and pods up to a few metres thick consisting mainly of pegmatitic green pyroxenes also occur in these old gneisses, although metadykes have not been observed to cut the ultrabasic pegmatites. Although the mineralogy of the leucocratic gneiss indicates a broad similarity to gneisses of igneous origin, no conclusive evidence bearing on the origin of these rocks has so far been recognised and they could represent acid-intermediate supracrustals metamorphosed in plutonic conditions.

Deformed varieties of the old gneisses in association with occasional graphite-bearing and sulphide-bearing garnet-sillimanite gneisses, which contain thin concordant ultramafic layers that may represent metadykes, form the rocks in which the layered intrusions were emplaced².

Rocks affected by the 2,800 Myr granulite facies metamorphism in which the metadykes and their deformed equivalents have not been recognised include the widely distributed layered cumulitic igneous sheets and abundant quartzofeldspathic gneisses which commonly retain intrusive relationships with the layered basic sheets and all the older rocks.

Three distinct intrusive phases of gneisses younger than the layered intrusions have been recognised. The earliest is a dark, intermediate phase which, although relatively uncommon and without convincing intrusive relationships, contains fragments of the older rocks, and has nowhere been found to contain early metadykes. The younger phase, intrusive into the first phase, is a light granitic rock which locally retains a coarse, probably igneous texture. Pink perthite-quartz veins and discordant sheets up to about 1 m thick constitute the last

intrusive phase. They occur throughout the complex but seem commonest at the margins of, and within, the layered basic rocks; sheets within the layered basic intrusions are usually thinner than the same bodies in the nearby gneisses. Although many of the younger phases indicate injection of the new material into the older rocks, at sites of locally high strain pegmatitic patches of the younger phase are commonly in close association with the old gneisses which contain the early metadykes.

All of these rocks show the effects of the overprinted, Scourian granulite facies metamorphism and must, therefore, be older than, or contemporaneous with, that event. North of Loch Laxford the Lewisian Complex has also suffered Laxfordian amphibolite facies metamorphism⁴ which affects the Scourie dykes. The effects of Scourian granulite facies metamorphism are, however, still occasionally retained. The younger phases of quartzofeldspathic gneisses north of Loch Laxford contain, and are intrusive into, fragments of layered basic rocks and supracrustal rocks, and in the field they are in every way comparable with the main younger intrusive phase in the granulite terrain south of the Laxford Front. The youngest phase is also represented north of the Laxford Front, as pink microcline-quartz sheets in the remnants of the layered basic rocks.

The oldest gneisses make up less than 50% of the complex between Badcall to Scourie, and occur as thin strips, up to about 100 m wide in the younger phases. The strips are often directly in contact with layered basic igneous sheets and are conformable with the structure marked by them. The younger, intrusive, quartzofeldspathic phases occur as veins and discordant sheets in all the older rocks and as large units which contain isolated, disrupted inclusions of the older rocks. The impression is that the strips of old rocks are seen in interrupted stages of incorporation into the younger phases. The oldest gneisses (containing metadykes), have not been recognised north of the Laxford Front and their scarcity or absence in that region could explain some of the geochemical differences known between the Scourian and Laxfordian complexes⁵. Major

bodies of successive younger intrusive phases are often located along interfaces between the older rock types, especially at the interface between units of old gneisses and of gneisses of supracrustal origin. Although the integrity of the ancient interface remains, marked by the layered basic sheets, the older gneiss has been almost completely incorporated into the younger gneisses. The long-lived influence of that ancient interface⁶ probably represents the junction between an extremely ancient basement (pre-Scourian) of old gneisses containing metadykes and the supracrustal rocks.

The field evidence suggests that gneisses younger than the layered basic intrusions have been derived both from injected material and from the local remobilisation and incorporation of gneisses older than the layered intrusions. The sequence of pre-2,800 Myr events (Table 1) is directly comparable with that determined for the western Greenland and Labrador portions of the North Atlantic craton^{7,8}. The gneisses older than the layered intrusions are comparable with the Amitsoq gneisses and those younger than the layered intrusions with the Nuk gneisses of the Godthåb region, western Greenland.

Table 1 Outline sequence of Archaean events in the Scourie region

- 8 Intrusion of Scourie Dykes about 2,200–2,400 Myr BP.
- 7 Deformation and amphibolite facies metamorphism commencing about 2,600 Myr BP.
- 6 End of granulite facies metamorphism, about 2,800 Myr BP.
- 5 Younger gneiss group:
 - iii Pink perthite-quartz veins common near contacts of layered bodies. Probably syntectonic to earliest recognised structures developed on Laxford lineament.
 - ii Light coloured homogeneous quartzfeldspathic gneisses. Main intrusive phase, igneous textures probably locally preserved.
 - i Dark coloured intermediate phase. Relationships uncertain but metadykes not found.
- 4 Layered cumulitic igneous sheets of the type associated with stratiform anorthosites of Outer Hebrides.
- 3 Metamorphosed dykes. Ultramafic varieties up to tens of centimetres wide.
- 2 Supracrustal rocks ranging from garnet-sillimanite gneisses and quartz-magnetite rocks to basic amphibolites.
- 1 Older gneiss group; quartzfeldspathic gneisses, intermediate pyroxene gneisses and ultramafic pods.

The recognition in the granulite facies terrain south of the Laxford Front of gneisses whose field relationships and relative ages resemble those of the Amitsoq group is in apparent conflict with strontium and lead isotope studies⁹ which suggest that the 2,800-Myr old terrain of the Lewisian Complex does not incorporate a significant proportion of reworked older crust (100–200 Myr old) equivalent to the 3,700-Myr old gneisses of western Greenland. These studies have led to the idea that a significant addition of crustal material from an upper mantle source, in the form of volcanic or plutonic rocks, occurred about 3,000–2,800 Myr BP in the Lewisian Complex. The recognition of old gneisses separable in the field from several generations of intrusive gneisses in the rocks of the granulite facies, and the possible derivation of younger gneisses by the local regeneration of old gneisses, do not support this postulation.

The absence of dates significantly older than 2,800-Myr and the isotopic ratios recorded from Lewisian rocks, could be partly explained by the ubiquitous nature of the younger intrusive phases, the youngest phase of which may be almost contemporaneous with the granulite facies metamorphism. Since those rocks form more than half the complex and provide most of the relatively homogeneous material suitable for sampling, the results obtained may be based mainly on an analysis of them alone.

To assist in future correlation between these units and others throughout the craton, the older gneisses and the supracrustal rocks are here respectively termed (after localities) the 'Uamhaig Gneisses' and the 'Claisfearn Supracrustals'. The metadykes are termed the 'Shios Dykes' and the suite of younger intrusive gneiss phases are termed the 'Clach Boga Gneisses'.

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Arctic palaeosalinities during late Cainozoic time

DURING the late Cainozoic, alternations between interglacial and glacial conditions, manifested by waning and waxing of continental ice sheets coincided with the rise and fall of seawater temperatures. Our palaeontological and oxygen isotope data indicate that although the major oceans and low latitude seas underwent large temperature variations, ~ 5–10 °C, Arctic water temperatures remained near freezing point for at least the past 3 Myr, the time interval represented by the longest studied cores. We have determined palaeotemperatures from the ratio of left to right coiling *Globigerina pachyderma*^{1–3} and calculated palaeosalinities using the oxygen isotope palaeotemperature expression of Epstein *et al.*⁴.

Our data suggest that normal and low salinity cycles were superimposed on temperature fluctuations. The lowest temperatures prevailed during the cold pulses of the Brunhes normal polarity and late Matuyama reversed polarity epochs and, somewhat higher temperatures and lower salinities a little more than about 1 Myr ago, in Matuyama time are indicated by planktonic foraminifera. Ice-rafted detritus throughout the Alpha Rise cores (Fig. 1) suggests that high latitude glaciation was under way 3 Myr ago. Ages have been estimated by interpolations and extrapolations based on radiometric dates and on close interval biostratigraphic and lithologic correlations between cores, some of which already have an established palaeomagnetic stratigraphy^{3,5}.

On the basis of faunal and sedimentary characteristics three major climatic units can be distinguished: Unit III include sediments deposited earlier than 2.4 Myr BP, during the Gauss reversed polarity epoch; the fauna constitutes less than 1% of the coarse fraction (> 63 µm, Fig. 2). Planktonic foraminifera are dominated by sinistrally coiled *G. pachyderma* (94–100%), and related forms, some of which are corroded^{3,5}. Arenaceous foraminifera dominate the benthonic foraminiferal assemblages. Sedimentation rates which were lower than those of the present day, and/or corrosive deep water may account for the selective solution of the less resistant calcareous tests. The impoverished character of the fauna precludes a palaeoclimatic reconstruction of this period.

The boundary between units II and III is marked by simultaneous lithological and faunal changes and occurs near a magnetic reversal (ref. 5; Fig. 2). The lithological change from dark brown, Mn and Fe oxide-rich, silty-lutite below, to tan silts with higher percentages of coarse, ice-rafted detritus above the boundary, is accompanied by faunal alterations. Sediments of unit II deposited about 2.4–0.7 Myr ago, during the Matuyama reversed polarity epoch, are poor in both foraminifera and in Fe and Mn oxides, but contain one layer rich in

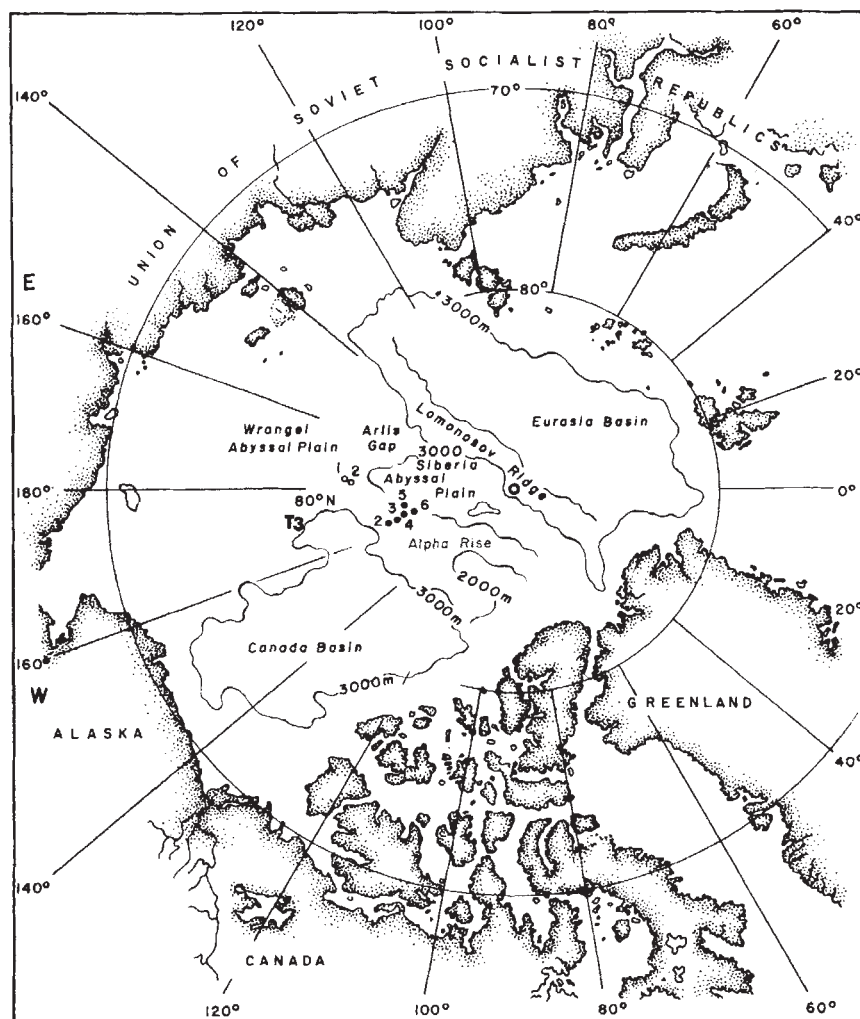


Fig. 1 Bathymetric map of the Arctic Ocean based on the *Geological Map of the Arctic* (1960). T3, Location of cores T3/67-3, 4, 9, 11, 12; PM, cores with know palaeomagnetic stratigraphy; RI, Radioisotopically dated core. Dark and open circles, locations of previously studies cores (refs 3 and 5).

Table 1 Data from collected fauna

Cores	Depths (cm)	Species analysed	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	Temperature** (°C)	Salinity ‰	$\delta^{18}\text{O}$ water	Melt water (%) ($\delta^{18}\text{O} = -30$)
T3/67-3*	98-101	<i>Cibicides wuellerstorfi</i> <i>Eponides tener umbonatus</i>	+3.93	+0.89	-0.5	33.8	-0.8	2.5
T3/67-3	98-101	<i>G. occlusa</i>	+3.16	+0.64	-1.7	32.5	-1.9	6.3
T3/67-3	98-101	<i>G. pachyderma</i>	+3.24	+0.78	-1.7	32.6	-1.8	6
T3/67-3	160	<i>G. pachyderma</i>	+1.00	-2.92	-1.0	30.4	-3.7	12.3
T3/67-3	160	<i>G. occlusa</i>	+1.30	+0.29	-1.0	30.8	-3.4	11.3
T3/67-3	160	<i>G. pachyderma</i> (5 chambered)	+0.96	+0.30	-1.5	30.0	-4.0	13.4
T3/67-3	203-205	<i>G. quinqueloba</i>	+0.17	-0.86	-1.5	29.1	-4.82	16.1
T3/67-3	219-222	<i>G. pachyderma</i>	+0.89	+0.30	-1.5	29.9	-4.1	13.7
T3/67-3	219-222	<i>G. occlusa</i>	-1.05	-1.46	-1.0	26.2	-7.3	24.4
T3/67-3	219-222	<i>G. pachyderma</i> (5 chambered)	+0.73	+0.31	-1.5	29.8	-4.2	14.2
T3/67-3	219-222	<i>C. wuellerstorfi</i>	+2.60	-0.04	-0.8	32.2	-2.2	7.3
T3/67-3	245-248	<i>G. pachyderma</i>	+1.12	+0.15	+1.5	31.5	-2.8	9.3
T3/67-3	245-248	<i>G. occlusa</i>	+0.73	+0.16	+1.5	30.9	-3.3	11.0
T3/67-3	250	<i>G. occlusa</i>	+1.15	+0.01	+1.5	31.5	-2.8	9.3
T3/67-3	250	<i>G. pachyderma</i>	+1.26	+1.5	+1.5	31.6	-2.7	9.0
T3/67-4†	80	<i>G. quinqueloba</i>	+1.42	-0.42	-1.0	30.6	-3.5	11.9
T3/67-11‡	Top	<i>G. pachyderma</i>	-1.21	-0.19	-1.5	27.5	-6.2	20.7
T3/67-11	6	<i>G. pachyderma</i>	+1.65	+0.70	-1.8	30.6	-3.5	11.7
T3/67-11	10	<i>G. pachyderma</i>	+0.62	+0.45	-1.0	30.2	-3.9	13.0
T3/67-11	10	<i>G. pachyderma</i>	+1.26	+0.01	-1.7	30.3	-3.8	12.7
T3/67-12§	0-2	<i>G. pachyderma</i>	+0.16	-0.04	-1.0	29.4	-4.6	15.6
T3/67-12	192	<i>G. quinqueloba</i>	-0.03	-0.95	-1.0	29.0	-4.8	16.3
Dst A2¶	0	<i>G. pachyderma</i>	+1.42	+0.56	-1.0	30.6	-3.5	11.9
Dst A2	7	<i>G. pachyderma</i>	+2.29	+0.67	-1.7	31.5	-2.7	9.3
Dst A2	39-40	<i>G. pachyderma</i>	+1.95	+0.82	-1.7	31.0	-3.1	10.5
Dst A2	75	<i>G. pachyderma</i>	+2.21	+1.10	-1.7	31.6	-2.8	9.0
Dst A2	180	<i>G. quinqueloba</i>	+1.15	-1.26	+1.5	31.4	-2.8	9.5
Dst A2	180	Composite sample	+1.42	-0.42				

* T3/67-3; 79°11'N; 175°09'W; 2,285 m water depth.

† T3/67-4; 79°22.7'N; 174°46'W; 1,760 m water depth.

¶ Dst A2; 83°52'N; 168°12'W; 1,521 m water depth.

|| 180 cm composite sample: Planktonic, benthonic foraminifers, ostracods, pelecypods.

** Temperature estimated mainly from the coiling direction of *G. pachyderma*; see text for details.

‡ T3/67-11; 79°34.9'N; 172°30'W; 2,810 m water depth.

§ T3/67-12; 80°21.9'N; 173°33'W; 2,867 m water depth.

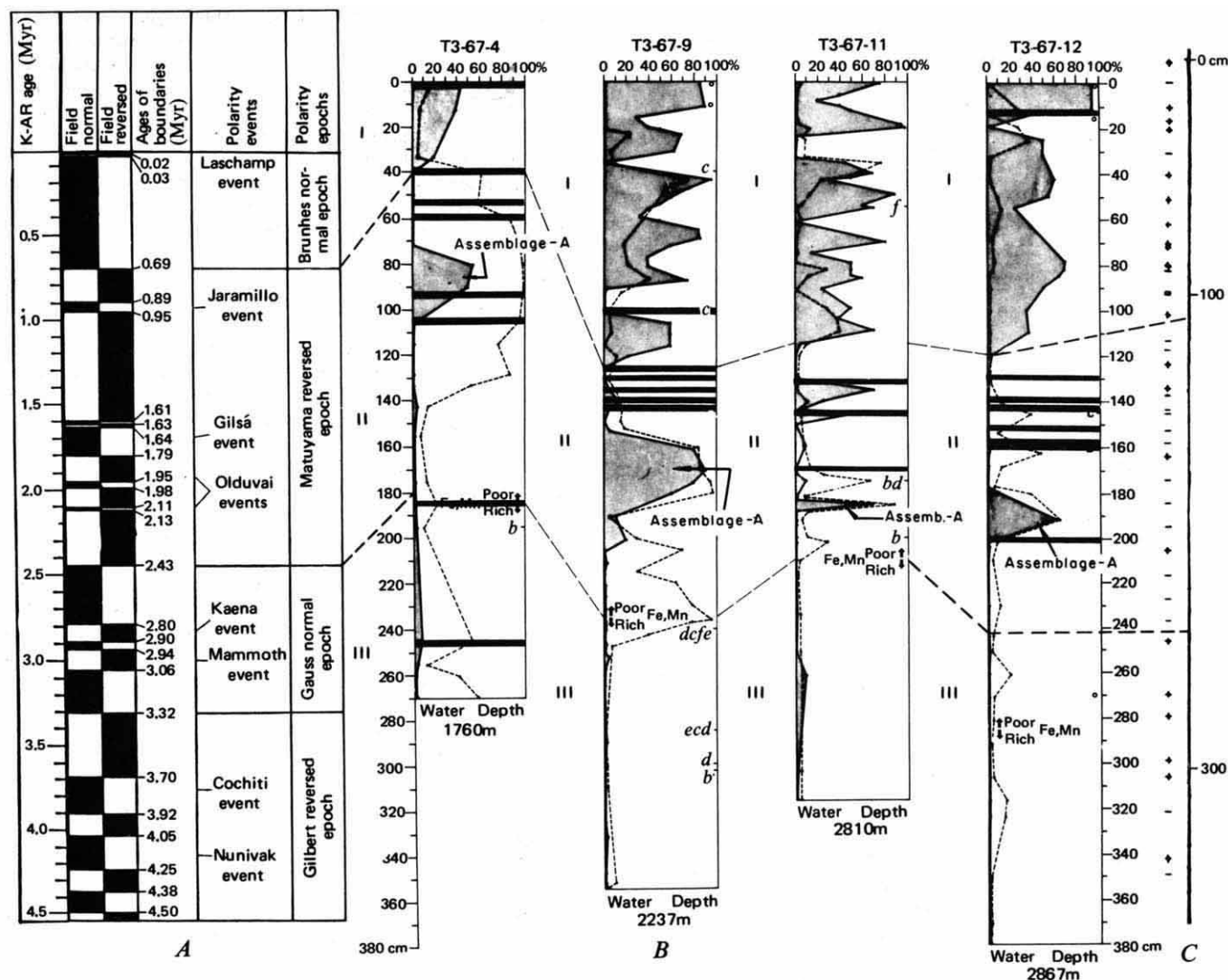


Fig. 2 A, Palaeomagnetic time scale after ref. 15. B, Ratio of microfauna to clastics in the coarse fraction of cores T3/67-4, 9, 11 and 12. Shaded areas, microfauna, white areas, clastics. Occurrence of low latitude foraminifera: a, *Globorotalia menardii-tumida*; b, *Globorotalia scitula*; c, *Globorotalia* sp.; d, *Globigerinoides* sp.; e, *Globigerinoides ruber*; f, *Sphaeroidinella dehiscentis*. Circles; Pteropods; Dark horizontal lines; zones in which benthonic foraminifera are in excess of 10% (assemblage B); dashed line, percentage of *G. quinqueloba* complex (including *G. exumbilicata*) out of the total planktonic foraminiferal population. C, Magnetic polarity changes in core T3/67-12 (ref. 16). Modified after ref. 3.

foraminifera. Two faunal assemblages, A and B, can be distinguished in this unit (Fig. 2).

In assemblage A, planktonic foraminifera constitute > 50% of the foraminiferal fauna. The relative abundance of *Globigerina exumbilicata* and *Globigerina quinqueloba* is high in shallow cores (< 2,000 m) and decreases considerably in deeper cores (Fig. 2), suggesting that their low frequency is attributable to solution. Data on their present-day distributional patterns and $^{18}\text{O}/^{16}\text{O}$ -determinations indicate that both *G. quinqueloba* and *G. exumbilicata* are eurytherm and euryhaline species^{3,5}. Low surface water salinities are believed to have determined the dominance of these two species during the Matuyama epoch. *Globigerina pachyderma* was not able to proliferate in water of low salinity, whereas *G. quinqueloba* and *G. exumbilicata* adapted to the new environment more readily. Occasionally low latitude foraminifera were observed^{3,5}; assemblage A was deposited during mild intervals of moderate to low salinities. In assemblage B, benthonic deep water, calcareous species, frequently accompanied by shallow water *Elphidium* spp., constitute up to 97% of the foraminiferal fauna⁵. The dominant planktonic taxa in these zones are *G. quinqueloba* and *G. exumbilicata* (Fig. 2). Dextral *G. pachyderma* and related forms attain highest frequencies in Matuyama sediments (ref. 3, Fig. 3). Assemblage B was probably deposited during warmer episodes

than assemblage A. Increased air temperatures enhanced glacier and shelf-ice melting into the Arctic Ocean and caused a further decrease in surface water salinities, leading to reduced planktonic productivity and a consequent dominance of benthonic foraminifera.

The climatic boundary between units I and II, defined by faunal and lithologic changes, corresponds approximately to the last major polarity reversal of the Earth's magnetic field, marking the Brunhes-Matuyama boundary (Fig. 2)^{3,5}. The sediments of Units I were laid down over the past 0.7 Myr, during the Brunhes normal polarity epoch, a time of conspicuous climatic fluctuations, as indicated by temporal variations in the faunal composition and in the fauna-mineral ratio (refs 3 and 5; Figs. 2-4). Foraminifera-rich and foraminifera-poor beds alternate with one another, with the former representing conditions similar to those prevailing today (permanent ice cap) and containing cold water, sinistral *G. pachyderma* and related forms almost exclusively. But *G. quinqueloba* and *G. exumbilicata* attain high frequencies at the onset and end of some of these cold periods (refs 3 and 5; Figs. 2 and 4), and the percentage of dextral, warm-water tolerant, *G. pachyderma* is generally highest in those zones (Fig. 3)⁵. This suggests a temporary warming with a subsequent increased supply of river and glacial melt water, followed by

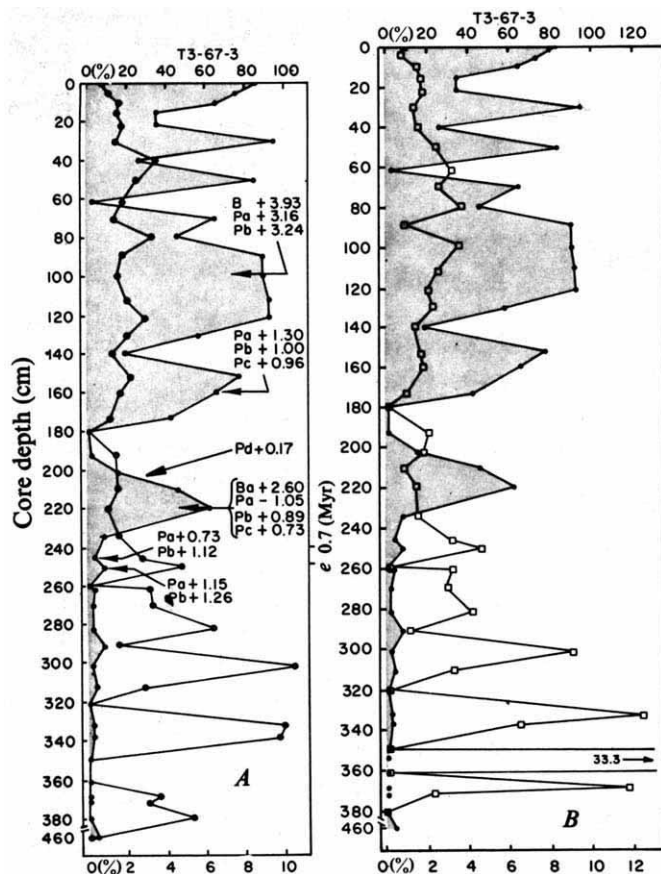


Fig. 3 Core T3/67-3. A, Ratio of microfauna to clastics in the coarse fraction (> 62 μm). Shaded areas, microfauna; white areas, clastics; values indicated on the abscissa at top. Occurrence of low latitude foraminifera: e, *Globigerinoides ruber*. Circles, percentage of dextral *G. pachyderma*; values indicated on abscissa at bottom. B, Same legend as for A. Squares, percentage of dextral *G. occlusa*. $\delta^{18}\text{O}$ values: B, benthonic foraminifera; Ba, benthonic foraminifera *C. wuellerstorfi*; Pa, *G. occlusa*; Pb, *G. pachyderma*; Pc, *G. pachyderma* (5 chambered); Pd, *G. quinqueloba*. Modified after ref. 3.

the formation of low-salinity surface water to which euryhaline-eurytherm *G. exumbilicata* and *G. quinqueloba* were able to adapt best^{3,5}. In the foraminifera-poor beds which we believe represent periods free, at least seasonally, of packice, the dominant, sinistral, *G. pachyderma* and related forms are accompanied by *G. quinqueloba* and *G. exumbilicata*^{3,5}. Occasionally, low latitude foraminifera occur^{3,5}.

Among the factors conditioning and modifying surface water salinity in the Arctic Ocean, precipitation, river runoff, evaporation, inflow and subsequent mixing of Atlantic and Pacific water, convective mixing, and the presence of driftice are considered important. The oxygen isotopic composition of Arctic Ocean surface water is determined by the same factors; evaporation results in an increase of ^{18}O whereas precipitation and river inflow causes its decrease⁶. At the present time the ranges of the temperatures and salinities of surface water are $-1.6-0^\circ\text{C}$ and $<29-34\text{‰}$, respectively^{7,8}. As a result of slow vertical mixing, present-day surface salinities are about 4.8‰ lower than those of the bottom water⁹. Oxygen isotope analysis of Arctic Ocean water indicates that the $^{18}\text{O}/^{16}\text{O}$ ratio closely follows the salinity profile, with $\delta^{18}\text{O}$ decreasing by 0.8‰ with a 1‰ decrease in salinity in the upper 350 m (ref. 9). The $\delta^{18}\text{O}$ value of this surface seawater is about 4‰ lower than at greater depth, where the $\delta^{18}\text{O}$ approaches values close to 0.

The $^{18}\text{O}/^{16}\text{O}$ ratio in the carbonate tests of planktonic foraminifera has been widely used to calculate past sea-surface temperatures and glacial ice volumes. The $\delta^{18}\text{O}$

values of calcareous tests depend on both temperature and the isotopic composition of the ocean. We estimated the palaeotemperatures by using the known correspondence between the coiling direction of *G. pachyderma* and present-day temperature zones in the world's oceans¹⁻³. With these temperature estimates and assuming a δ value of -30‰ for glacial melt water, we calculated the palaeosalinities using the oxygen isotope palaeotemperature expression of Epstein *et al.*⁴ (Table 1).

We have measured the $\delta^{18}\text{O}$ of foraminiferal tests from representative foraminifera-rich and foraminifera-poor layers in five cores (Table 1) (for results based on earlier determinations see ref. 3). The foraminifera were hand picked, treated with commercial Clorox solution to remove organic contaminants and then reacted with 100% H_3PO_4 according to the method of McCrea¹⁰. The liberated CO_2 was analysed for carbon and oxygen isotope ratios mass spectrometrically. We found that between the depths of 250 and 160 cm in core T3/67-3, deposited during the late Matuyama and early Brunhes (a time of relatively mild climates when the Arctic is thought to have been at least seasonally ice free, and freshwater dilution of the surface layer would have brought about density stratification), the $\delta^{18}\text{O}$ values of planktonic foraminifera differ significantly from those of the benthonic forms (ref. 3, Table 1; Figs 3 and 4). The density stratification would have been maintained for as long as the freshwater supply prevented sinking of the light, low salinity, surface water^{3,5}. On the other hand, during the cold pulses of the Brunhes epoch, represented by the foraminifera-rich zones, when the presence of permanent packice increased the salinity and thus the density of the surface layers, the $\delta^{18}\text{O}$ values of the surface water approached the values of the deep water (ref. 3; 98–101 cm level in T3/67-3, Table 1; Figs 3 and 4).

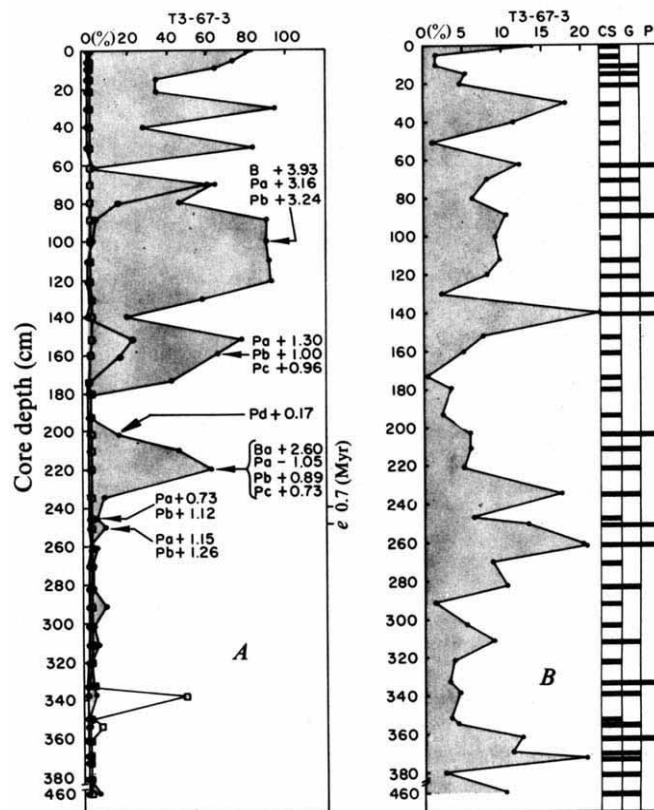


Fig. 4 Core T3/67-3. A, Ratio of microfauna to clastics in the coarse fraction (> 62 μm). Shaded areas, microfauna; white areas, clastics; open circles, percentage of *G. quinqueloba* of the total adult planktonic foraminiferal population; open squares, percentage of *G. exumbilicata* of the total adult planktonic foraminiferal population. B, Percentage of coarse fraction (> 62 μm). CS, coarse sand; G, granules; P, pebbles. $\delta^{18}\text{O}$ values: B, benthonic foraminifera; Ba, benthonic foraminifera *C. wuellerstorfi*; Pa, *G. occlusa*; Pb, *G. pachyderma*; Pc, *G. pachyderma* (5 chambered); Pd, *G. quinqueloba*. Modified after ref. 3.

Our data, together with the results of other investigators^{3,5,11-14}, suggest that the Matuyama epoch was a time of equable and higher global temperatures than the preceding late Gauss and the following early Brunhes^{5,11-14}. As long as large volumes of glacier ice calved and melted into the Arctic Ocean during the worldwide climatic amelioration, the water did not warm up appreciably. The main result of cold, fresh water being discharged into the Arctic Ocean was the freshening of surface water. Consequently, faunal changes probably reflect primarily the effects of salinity oscillations rather than temperature changes. It is assumed that during most of the Matuyama epoch the Arctic Ocean was free at least seasonally of permanent packice, so that ice laden with debris drifted unimpeded across the ocean, melting and releasing to the sea floor the incorporated detritus. The amplitudes of global temperature fluctuations were larger during the Brunhes epoch than in the Matuyama epoch^{3,5,12-14}. In the Arctic, these cold-warm pulses are expressed by the alternations of foraminifera-rich and foraminifera-poor zones, which may correspond to the mid-latitude, major, glacial-interglacial cycles.

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Electrical properties of dry and humid sand

ALTHOUGH heterogeneous, sand behaves in many ways like a typical inorganic solid. Its natural abundance makes its electrical properties important in radio propagation and in electromagnetic geological and hydrological surveys, for example using the wave-tilt technique¹. We have undertaken an experimental study of the complex permittivity (ϵ' , ϵ'') or conductivity of various natural sands at laboratory temperature in controlled humidities, over the frequency range 0.1 to 5×10^6 Hz ($T=20 \pm 1^\circ\text{C}$).

A nickel-plated brass three-terminal cylindrical cell was used, with internal diameter 3.0 cm, external diameter 4.0 cm, length 4.12 cm, guard ring length 2.0 cm, guard ring spacing 0.5 mm. It was mounted in a vacuum system of ultimate pressure $\sim 10^{-2}$ mmHg, and measurements were made for several days until they did not alter, it being then assumed that the sand specimen was completely dry. Preheating *in vacuo* did not change the data. Measurements were then made with the specimen quickly transferred to a controlled humidity oven, where it was exposed either to silica gel or to saturated solutions

Table 1 Limiting permittivities, relaxation times and spreading parameters from conventional analysis

Humidity %	ϵ_s	ϵ_∞	τ (s)	α
94	1.65×10^5	3.94	2.56	0.54
74	1.36×10^4	3.16	11.1	0.46
33	240	2.73	90.9	0.42

of inorganic salts⁵. Further measurements on the dry specimens were taken in the vacuum system itself, and agree with those taken at zero humidity in the oven. The computer-controlled frequency response analyser system of Chelsea College was used at $< 10^4$ Hz; in the range 10^4 – 10^6 Hz the General Radio 1621 precision capacitance measurement system was used, and in the range 10^6 – 5×10^6 Hz the Wayne Kerr bridge and system SR 268 were used. There were no significant systematic discrepancies in the regions of overlap.

The overall accuracy is $\pm 0.1\%$ in ϵ' and ϵ'' , but the state of packing of the sand is less repeatable ($\pm 1.0\%$).

Sand specimens were taken from desert regions of Persia and Libya, and from the seashore in Derrynane, Ireland, and Sennen Cove, England; all were thoroughly washed before measurement, and sieved where necessary. The differences between data for different sands are reasonably small ($\leq 10\%$). Figure 1a and b represents the data for Libyan sand, which are typical.

To our knowledge, in none of the previous electrical experiments on sand has absorbed water been shown to be absent:

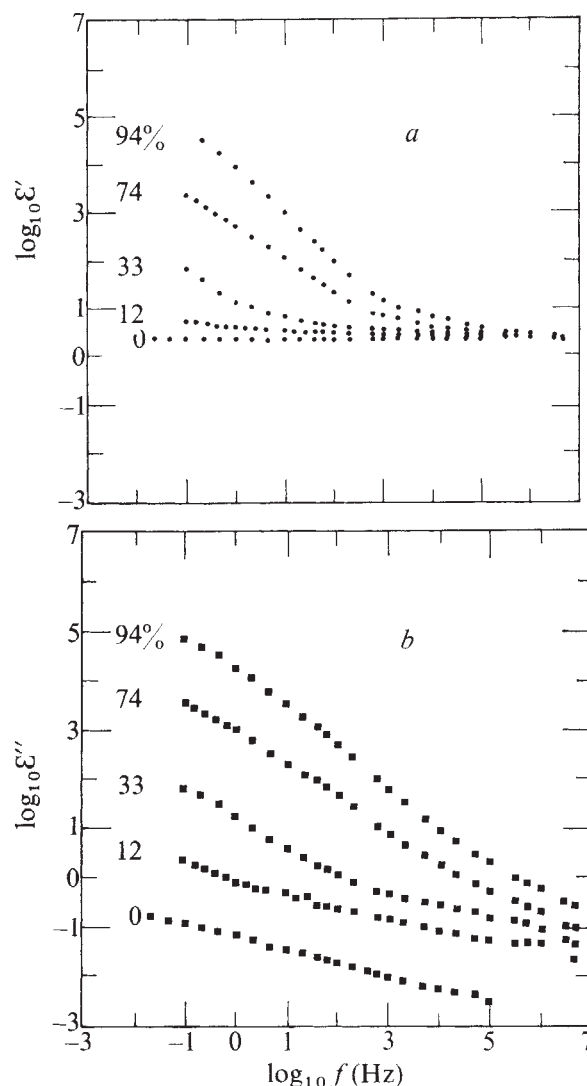


Fig. 1 a and b, Complex permittivities ϵ' , ϵ'' of Libyan sand, as a function of frequency f , plotted logarithmically at various percentage humidities, as indicated.

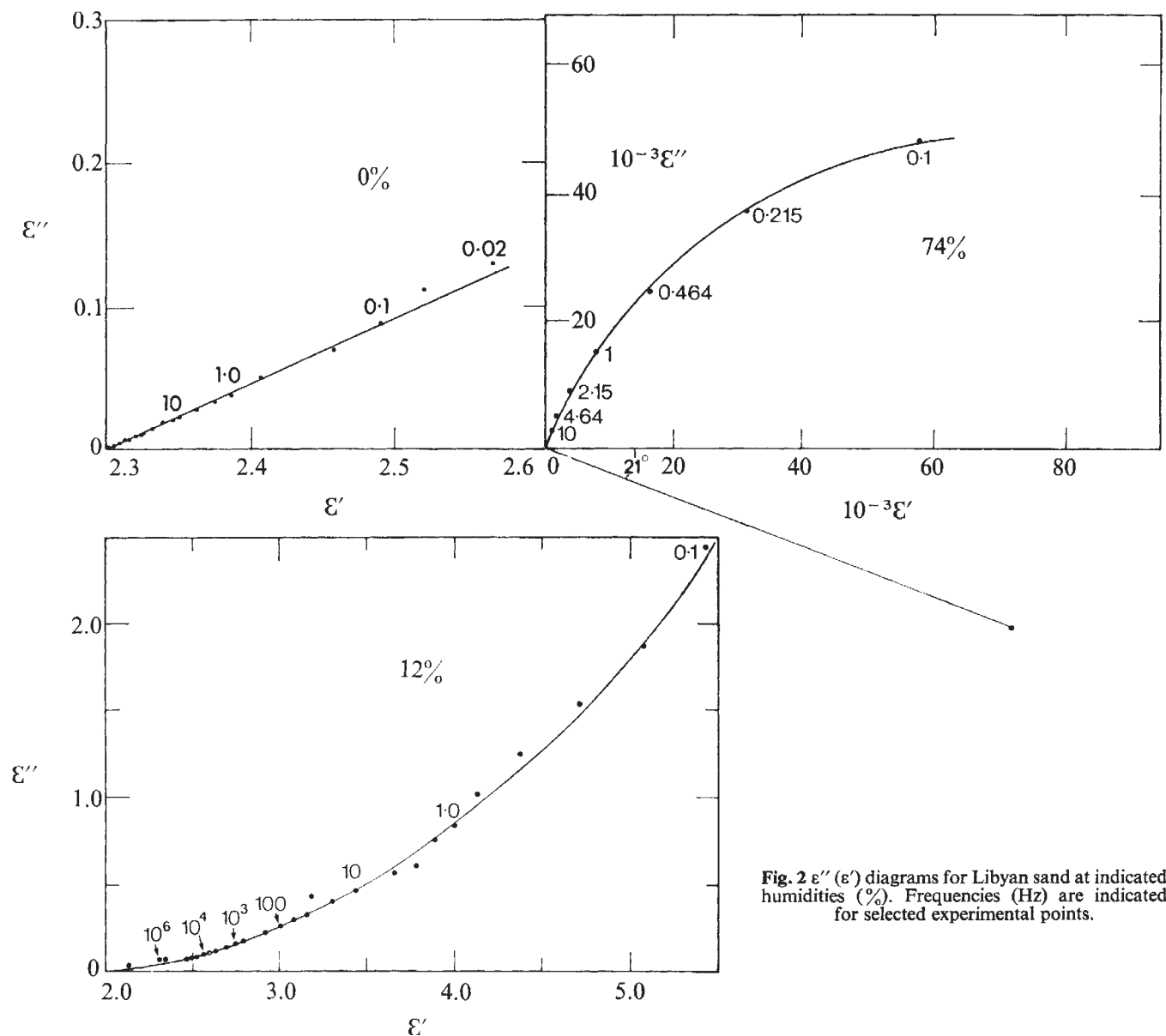


Fig. 2 $\epsilon''(\epsilon')$ diagrams for Libyan sand at indicated humidities (%). Frequencies (Hz) are indicated for selected experimental points.

laboratory exposure of the specimen after heating and vacuum drying markedly alters the electrical properties within minutes. The dry sand behaves in the manner noted and interpreted by Jonscher² as being common to many bulk dielectrics, in that both $\epsilon' - \epsilon_\infty \propto \omega^{n-1}$ and $\epsilon'' \propto \omega^{n-1}$, where in the present instance $n = 0.77$, and ω is the pulsance. This behaviour is characteristic of 'screened hopping' or jumping of either charge or dipoles, with the screening charge continually adjusting to the time-averaged occupancies of the sites.

Sand is a heterogeneous mixture of crystalline silica and air, and one might therefore expect dielectric mixture equations³ to be applicable. The situation for dry sand will be qualitatively the same as that for bulk natural quartz.

For the humid sand, however, real permittivities as much as five orders of magnitude larger are found at low frequencies, which arise from the highly conducting surfaces of the grains. The specimen conducts electricity in the limit of low frequency; the d.c. conductivity is determined by drawing a horizontal tangent to the conductivity-frequency plot. This conduction corresponds to paths right across the specimen, which must be distinguished from conduction paths on grains which are insulated from each other. When the d.c. conductivity is subtracted from the data, the frequency dependence of the loss ϵ'' shows a broad maximum.

In the complex (ϵ' , ϵ'') plane (Fig. 2, humidity 74%) the characteristic depressed semicircular arc is displayed. Conven-

tional analysis leads to principal relaxation times τ_0 , Cole and Cole⁶ spread parameters α and limiting permittivities ϵ_s , ϵ_∞ , as shown in Table 1. The proportion of water by weight in this sand has been determined to be 2.34% at 94% humidity.

It is difficult to reconcile such a large spread of relaxation times with a distribution of shape factors in the Maxwell-Wagner process; we are therefore forced to conclude that there is a distribution of activation energies for surface conduction. This situation, for the counterions surrounding colloidal particles, has been treated by Schwarz⁴ who took into account the potential distribution produced on the spherical surfaces by diffusion and electrostatics. His treatment did not, however, include the more modern dielectric mixtures theories which are now available⁷. Analysis of the data with their aid is in hand.

The very large low frequency variation of permittivity presents difficult problems for the interpretation of radio propagation and electromagnetic hydrological and geological survey; it is expected that clays, soils and porous rocks will behave similarly, and experimental studies are being undertaken.

The dry sand demonstrates a completely different form of $\epsilon''(\epsilon')$ diagram (Fig. 2, 0%). Because of the proportionality of the energy loss per cycle to the energy stored per cycle², $(\epsilon' - \epsilon_\infty)/\epsilon''$ is frequency independent, and a straight line $\epsilon''(\epsilon')$ is found. At low humidities the ω^{n-1} component is raised (Fig. 1b) and the high frequency $\epsilon''(\epsilon')$ straight line form is enhanced by the semicircular arc which would dominate at

extremely low frequencies; the resulting form (Fig. 2, 12%) displays an upward curvature. Thus the shape of the diagram indicates the relative importance of the two polarisation mechanisms proposed. At high humidities (Fig. 2, 74%) a fully developed circular arc appears. The possible contribution of variation in n to this situation is not yet evaluated.

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Optical resolution by chromatography at low temperature

We report here the first successful optical resolution experiments using low temperature chromatography, for which we used the tris-acetylacetonato (acac) complexes of Al(III) and Fe(III). Since the apparently unsuccessful attempts to resolve Fe(oxalate)³⁻ at -30°C on starch¹, only room temperature experiments have been reported (for example, refs 2–4).

Using a high-pressure pump (20 MPa) the eluent 4:1 isopentane–ethyl ether, was used here, but 2 chlorobutane (or dimethyl ether could be used down to 140 K) was run ($0.5\text{--}2\text{ ml m}^{-1}$) through the column (diameter 7.5 mm, length 300 mm) enclosed in a thermostated Cu-block in a liquid N_2 cryostat. The sample (typically 0.05 ml of a saturated ethanol solution)

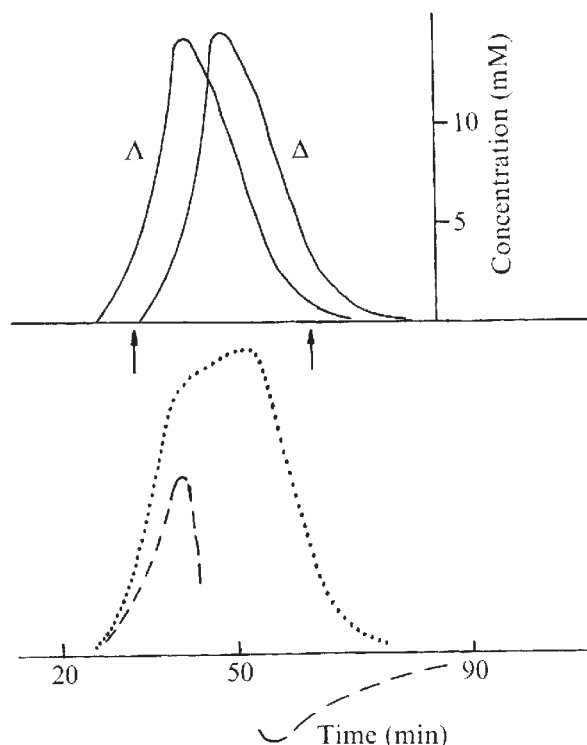


Fig. 1 A typical elution pattern, $\text{Al}(\text{acac})_3$. Absorbance (—) and circular dichroism (---) at 303 nm against the retention time and possible corresponding concentrations of the enantiomers.

was injected through a septum into the eluent line just outside the cryostat. The eluate was led from the column through an optical cell and then out of the cryostat. All optical windows were birefringence free.

The column filling was D-lactose and Al_2O_3 : a saturated solution of 4.5 g D-lactose was stirred with 10 g chromatographic Al_2O_3 (basic, 100–150 mesh). About 50% of the

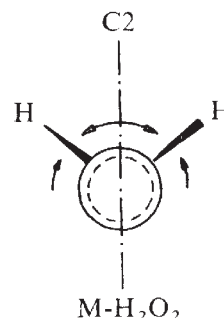


Fig. 2 The $\text{M-H}_2\text{O}_2$ molecule.

water was evaporated at 80°C , and the slurry obtained was mixed with 100 ml benzene. The liquid phase was removed with a glass filter, and the filtrate was dried with air at 70°C . Aggregation of the Al_2O_3 particles thus seemed to have been prevented.

A high optical yield was suggested by an almost constant ratio between the circular dichroism (CD) and the absorbance (A) of the early fractions after the breakthrough and in some cases also by a shoulder on the absorbance curve. $\text{Fe}(\text{acac})_3$ and $\text{Al}(\text{acac})_3$ could be resolved below -100°C and -30°C , respectively. By analogy with other $\text{M}(\text{acac})_3$ complexes on D-lactose, it is concluded that the enantiomer with the absolute configuration Λ (IUPAC nomenclature⁵) appears first from the column. The observed CD of the Fe complex was fairly diffuse (extremum at about 270 nm: $\text{CD}/A = +1.4 \times 10^{-4}$) while $\text{Al}(\text{acac})_3$ showed the two expected bands at 300 nm due to dipole couplings between locally excited transitions in the acac ligands: $\Delta\epsilon/\text{M}^{-1}\text{ cm}^{-1}$ (the wavelength of extremum in nm, assuming perfect optical purity, is in parentheses): -54 (276), $+115$ (303). The spectral details and the inversion kinetics of $\text{Al}(\text{acac})_3$ are to be discussed elsewhere⁶.

The use of optical activity methods would be vastly expanded if molecules could be resolved which are (statistically) inactive at room temperature but have asymmetric ground state configurations. Hydrogen peroxide (H_2O_2), for example, with O–O axis rotation barriers which are symmetric about the $\text{C}_2\text{--O--O}$ plane (see Fig. 2), should be a mixture of equal amounts of the two optical isomers M and P (for nomenclature see ref. 7) at low temperatures. Preliminary studies on its analogue S_2I_2 indicate that it may be resolved at 77 K by photolysis by circularly polarised radiation, as has been shown already for 1,2-dithiane⁸. With only four atoms this is the simplest conceivable optically active species.

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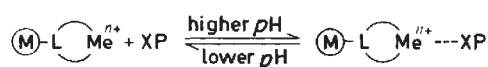
Metal chelate affinity chromatography, a new approach to protein fractionation

CONVENTIONAL nonspecific precipitation methods sometimes depend on affinities which can be used in a more selective fashion by modern chromatographic techniques. The affinity of proteins for heavy metal ions, for example, may provide a basis for their purification and analysis. A highly flexible method based on such affinities is described here.

Histidine and cysteine are known to form rather stable complexes with zinc and copper ions in nearly neutral aqueous solutions¹. A gel loaded with strongly fixed Zn^{2+} and Cu^{2+} ions with excess binding capacity may therefore interact with surface-exposed imidazole and thiol groups of proteins. Consequently zinc and copper-containing hydrophilic gels might be selective adsorbents for histidine and cysteine-containing peptides and proteins. The same should be true of gels and ions of transition elements such as Cd, Hg, Co and Ni, which can all form coordination compounds with histidine and cysteine. To test the validity of this idea, chelate-forming ligands for these metal ions were attached to highly permeable crosslinked agarose.

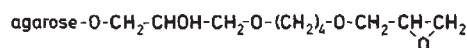
With new types of agarose derivatives² it is easy to synthesise chelate-forming adsorbents for proteins. If the metal is a transition element, the affinity will be pH dependent in such a way that selectivity for histidine, and presumably cysteine, will be favoured at the lower range (pH 6–8), where adsorption of proteins occurs. At alkaline pH, coordination with amino groups takes place, making the adsorption more effective but less selective.

To facilitate easy regeneration of the adsorbent for chromatographic purposes, metal ions must have a much higher affinity for the gel than for the substance to be separated. The principle is thus as follows

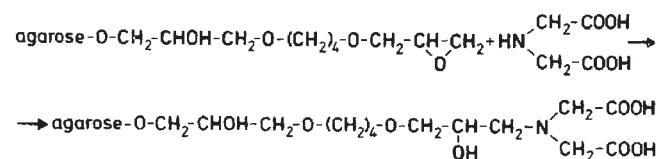


where M signifies the gel-forming matrix, L the chelate-forming ligand, Me^{n+} the metal ion, and X a metal affinity substituent in the protein or peptide XP.

For illustration we have selected serum proteins as XP, oxirane-activated agarose



as M, *bis*-carboxymethyl amino groups as L, and Zn^{2+} and Cu^{2+} as Me^{n+} . The chelate gel is synthesised according to the following scheme



Disodium salt of iminodiacetic acid (2 g) was dissolved in 10 ml of 2 M Na_2CO_3 and added to 15 ml of suction-dried oxirane-activated agarose (Sephacrose 4B) in a conical flask. The suspension was heated to 65 °C and stirred for 24 h. The gel was collected on a fritted-glass filter and washed with distilled water. The product, *bis*-carboxymethyl amino agarose (see reaction scheme above), contained 630 μmol of *bis*-carboxymethyl amino groups per g dry gel (25 μmol per ml swollen gel) as determined by nitrogen analysis according to the Kjeldahl method and titration of carboxyl groups.

Gel-bound chelates of zinc and copper were prepared by passing water solutions of zinc chloride (ZnCl_2 , 1 mg ml⁻¹) and

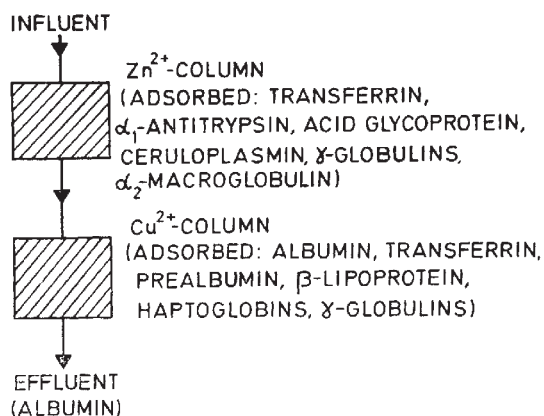


Fig. 1 Coupling of composite column.

copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 mg ml⁻¹) through columns (1×3.6 cm) containing the *bis*-carboxymethyl amino agarose. The Zn^{2+} column was completely saturated with Zn^{2+} , as shown by the presence of Zn^{2+} in the eluate, whereas the Cu^{2+} column was loaded with Cu^{2+} only to 60% of its length to avoid contamination of the eluate with Cu^{2+} . The chelate gel was able to adsorb about 20 μmol of Cu^{2+} per ml.

Human serum (2.7 ml) was fractionated by passing it through the two columns coupled in sequence, beginning with the Zn^{2+} column (Fig. 1); the two columns were then disconnected and each was eluted separately (Fig. 2). Neither Cu^{2+} nor Zn^{2+} was detected in the eluate until, in the final elution step, EDTA solution was passed through the columns (Fig. 2).

The fractions obtained were subjected to electrophoresis on gradient acrylamide gel plates (4–30%) in a Gradipore 342000 apparatus. A sample of 30 μl of the material with A_{280} of 1–3 was applied to the top of the plate and electrophoresis was run for 24 h in 0.089 M Tris-borate buffer (pH 8.3) containing 2.5 mM EDTA. The separated proteins on the gel plate were stained with amido black and destained with acetic acid (7%). The components tentatively identified are summarised in Table 1 (numbers indicate their relative order of appearance in the fraction).

The fractionation seems to be very sharp for many of the compounds and no signs of denaturation were observed. All electrophoretic bands in the original serum were detected in

Fig. 2 Composite chromatogram obtained by elution of the coupled Zn^{2+} and Cu^{2+} columns (a). The two columns were washed with 0.05 M Tris-HCl buffer, pH 8, 0.15 M in NaCl (60 ml each). The adsorbed material was removed from each column separately, according to the following scheme. Cu^{2+} column (b): 1, 0.1 M Na phosphate buffer, pH 6.5, 18 ml; 2, 0.1 M Na phosphate buffer, 0.8 M in NaCl, pH 6.5, 20 ml; 3, 0.1 M Na acetate buffer, 0.8 M in NaCl, pH 4.5, 17 ml; 4, 0.05 M EDTA, 0.5 M in NaCl, pH 7.0, 25 ml. Zn^{2+} column (c): 5, 0.1 M Na phosphate buffer, pH 6.5, 47 ml; 6, 0.1 M Na phosphate buffer, 0.8 M in NaCl, pH 6.5, 38 ml; 7, 0.1 M Na acetate buffer, 0.8 M in NaCl, pH 4.5, 12 ml; 8, 0.05 M EDTA, 0.5 M in NaCl, pH 7.0 10 ml.

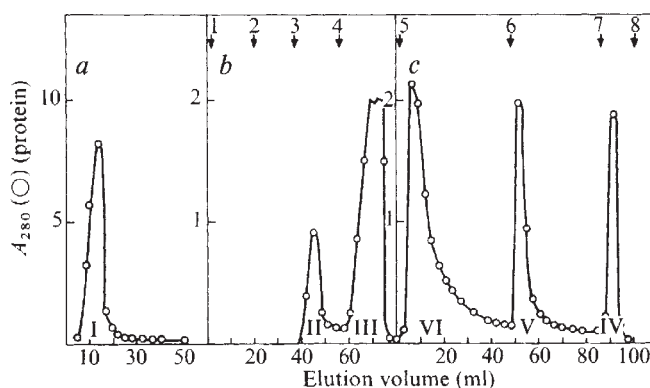


Table 1 Components identified by Gradipore electrophoresis of the fractions obtained in metal chelate affinity chromatography of human serum on Cu^{2+} column and Zn^{2+} column

	Peak	Components identified
Material that passed both columns in conditions of adsorption: 0.05 M Tris-HCl, pH 8, 0.15 M NaCl	I	Albumin
Material desorbed from Cu^{2+} column: Na acetate, 0.8 M NaCl, pH 4.5	II	Albumin, γ -globulins, prealbumin, traces of α_1 -antitrypsin
0.05 N EDTA, 0.5 M NaCl, pH 7.0	III	Albumin, transferrin, haptoglobins, β -lipoprotein, traces of γ -globulins
Material desorbed from Zn^{2+} column: Na phosphate, pH 6.5	IV	Transferrin, α_1 -antitrypsin, acid glycoprotein, γ -globulins, ceruloplasmin
Na phosphate, 0.8 M NaCl, pH 6.5	V	Transferrin, traces of haemoglobin*, traces of γ -globulin
Na acetate, 0.8 M NaCl, pH 4.5	VI	α_2 -Macroglobulin, traces of haemoglobin*

*Present as contaminant in the serum used.

the fractions and no new bands were observed. In the same starting buffer, but in the absence of heavy metal ions, very little protein was adsorbed. At 1 M NaCl the metal chelate gel was still an efficient adsorbent. The involvement of ordinary ionic adsorption in the fractionation is therefore ruled out.

Orienting experiments have shown that of the amino acids present in proteins, histidine and cysteine are most strongly adsorbed. Histidylhistidine is very strongly adsorbed at pH 7 as a consequence of the cooperative effect of the histidine residues. Other amino acids are also adsorbed at pH 8–9 but are desorbed far in advance of histidine when the pH is lowered. We therefore believe the chromatographic behaviour of a protein to be largely governed by its surface number or density of exposed imidazole and thiol groups. In addition, other groups such as indole may be important.

With the metal chelates we have tested, the adsorption capacity for serum proteins decreased in the following order: $\text{Cu} > \text{Zn} > \text{Ni} > \text{Mn}$, with Cu being very effective and the Mn gel having almost negligible adsorption capacity. There also seem to be qualitative differences that are worth exploring.

Metal affinity has been used to purify proteins containing or dependent on metal ions³; however, the method described here is more general in its application and not restricted to proteins. The potential advantages and limitations of this method will have to be explored further, but the capacity and separation power seem to make it suitable for large scale fractionations.

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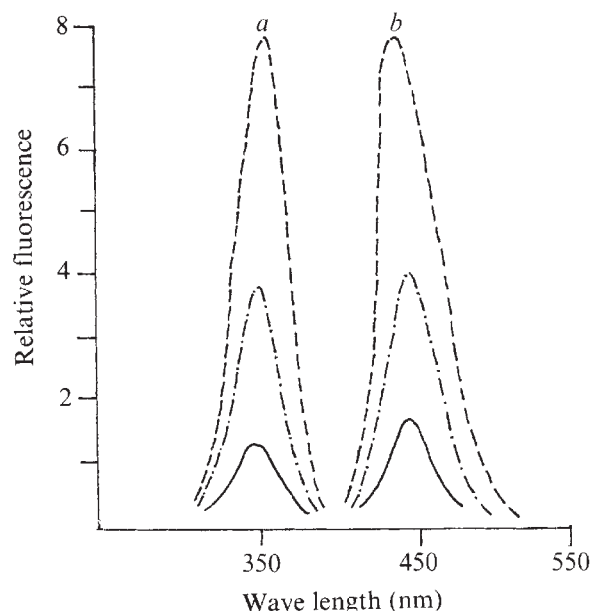
Fluorescent product accumulation in ripening fruit

STUDIES of mammalian tissue indicate that free radicals mediate fluorescent product accumulation^{1–3} and that such products are causatively related to cellular senescence^{4–7}. Fluorescent products isolated from senescent tissues, cells stressed by oxidants such as ozone, or cell-membrane isolates have characteristic fluorescence at 360–380-nm excitation and 440–470-nm emission⁸. These pigments have been identified in various mammalian tissues^{1–3}, protozoa¹¹,

nematodes¹², and insects^{8,13,14}. We are not aware of any reports of the occurrence of lipofuscin-like pigments in plant tissues. This study reveals the presence, in ripening fruit, of fluorescent material of spectral characteristics identical to lipofuscin from mammals. The pigment is present at greater concentration in a subcellular fraction than in the whole tissue and has a molecular weight in excess of 4,000. The yield of fluorescent product increases with ripening of banana and pear fruits.

Fluorescent products were extracted from the peel and pulp tissue of ripening banana (*Musa cavendishii*, var. Valery) and pear (*Pyrus communis*, var. Bartlett) by the procedure described by Fletcher *et al.*¹⁵. Lipofuscin isolates were water-washed and irradiated with ultraviolet light to remove possible interfering substances¹⁵. A crude mitochondrial fraction was isolated from banana pulp by the procedure outlined elsewhere¹⁶. Molecular weight was estimated with a 58×2.5 cm column packed with Sephadex LH-20 in chloroform-hexane (65:35, v/v). The eluant was monitored for fluorescence with a flow cell at 360-nm excitation and 440-nm emission. Fluorescence spectra were obtained with an Aminco-Bowman spectrophotofluorometer at 10 °C with 3-, 1- and 3-mm slits for the 3-, 4- and 6-slit positions. The machine was standardised with $0.1 \mu\text{g ml}^{-1}$ quinine sulphate in 0.1 N H_2SO_4 . The standard

Fig. 1 *a*, Excitation and *b*, emission spectra of chloroform-soluble lipofuscin isolated from ripening Bartlett pear fruit. ---, Over-ripe; - - - -, ripe; —, green fruit.



had a fluorescence intensity of 1.9 units at 360-nm excitation and 440-nm emission. The relative fluorescence units were derived from the following formula: (Meter multiplier value/100) $\times$ % fluorescence. The fluorometer blank was adjusted and solvent subtracted.

Excitation and emission scans of lipofuscin isolates from preclimacteric, climacteric and postclimacteric pear are illustrated in Fig. 1. These spectra are similar to those obtained from banana fruit and reported for mammalian sources of fluorescent product¹⁷. Yield of the fluorescent substance from banana pulp increased exponentially after treatment of green fruit with 100 p.p.m. ethylene (Fig. 2). A linear increase in fluorescent pigment occurred during peel ripening. Similar increases in pigment also occurred during ripening of pear fruits (Fig. 1).

The mitochondrial fraction isolated from postclimacteric pulp contained approximately sevenfold more lipofuscin than did the whole pulp tissue. The emission spectrum of mitochondrial lipofuscin was broader (peaking at 450 nm) than that for the whole tissue isolates. Banana mitochondria lipofuscin had spectra similar to those reported by Chio and Tappel for *in vitro* oxidised rat liver mitochondria⁴. Fluorescence yield from heavier and lighter sedimented cell fractions was similar to the whole tissue. The higher yield of fluorescence from the mitochondrial fraction may reflect the occurrence of lysosome-like particles containing membrane debris as indicated in electron microscopic analyses of ripening fruit¹⁸.

The fluorescent substance isolated from banana pulp eluted in the void volume of a Sephadex LH-20 column indicating a molecular weight in excess of 4,000.

These data support our contention that a lipofuscin type of pigment accumulates during ripening and proceeding senescence of fruit. The excitation and emission spectra of fruit lipofuscin are comparable with those reported for animal lipofuscin. The pigment is barely detectable in preclimacteric fruit and increases with ripening and postclimacteric development. The association of this pigment with a particulate fraction and the high molecular weight are also consonant with their lipofuscin identity. Our suggestion that peroxidative damage to cell membranes is a primary event of plant senescence is supported by other experiments. Cellular membranes from plant tissues contain relatively large amounts of polyunsaturated fatty acids in their amphipathic lipids. Ripening of fruit is associated with a decline in the highly unsaturated fatty acids of mem-

brane lipids^{19,20}; increased activity of various oxidative enzymes²¹⁻²³ and gross changes in membrane integrity^{24,25}.

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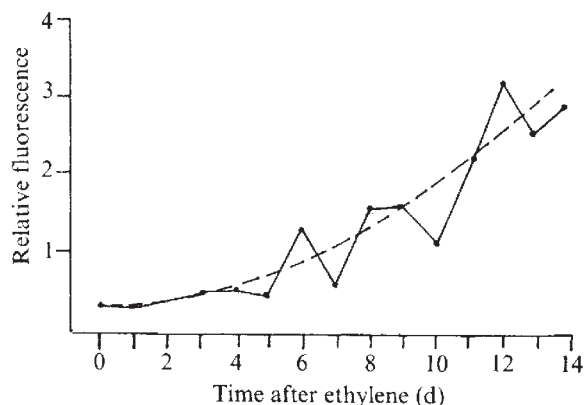
Lectins stimulate pollen germination

POLLEN germination involves the conversion of the quiescent vegetative cell into a metabolically active and rapidly growing pollen tube¹. Although many external factors influence pollen germination, no predominant control mechanism has been identified. In certain lymphocytes in culture, lectins (plant agglutinins) have been shown to stimulate quiescent cells to enlarge and synthesise DNA^{2,3}. Lectins also bind to the surface of actively growing cells and cause agglutination of red blood cells and transformed or malignant cells²⁻⁴, of gametes of *Chlamydomonas*⁵ and of carrot protoplasts⁶. There have, however, been few studies of the effects of lectins on plant tissues. The results presented here show that the lectins, concanavalin A (con A) and phytohaemagglutinin-M (PHA), stimulate pollen of *Lilium longiflorum* to germinate more rapidly than controls, by reducing the length of the lag period before pollen tube emergence.

Mature pollen from greenhouse-grown plants of *Lilium longiflorum* (cv Ace) was collected from anthers dehiscent at room temperature. The pollen was mailed from Urbana, Illinois to Berkeley, without special precautions and stored at 4 °C over Drierite. Samples (10 mg) of pollen were germinated in 50-ml flasks containing 2.5 ml of germination medium¹ (1.27 mM Ca(NO₃)₂ · 4H₂O, 0.99 mM KNO₃, 0.16 mM H₃BO₃, and 0.29 M sucrose) in a shaker water bath at 30 °C. Con A (Sigma: Grade IV, highly purified) and PHA (Calbiochem: B grade) were dissolved in the germination medium. In some experiments weighed pollen samples were humidified before germination in a closed container over water for 1 h (ref. 7). This procedure improved germination, especially in pollen which had been stored for one month or more. Aliquots of pollen were taken from the flasks at hourly intervals and the germinated grains were identified and counted microscopically.

In solution of 1 mg ml⁻¹ con A, the largest percentage of pollen grains germinated during the first hour in the germination medium, while that of control pollen grains germinated during the third hour (Fig. 1). In 100 µg ml⁻¹ con A, the maximum germination rate occurred during the

Fig. 2 Recovery of fluorescent products (chloroform soluble) from ripening banana fruit after treatment with ethylene. Day zero fruit were number 2 (full green pulp), day 5 were number 4 (yellow flush pulp), day 8 fruit were number 5 (full yellow with green tips) and day 14 were number 8 (brown spotted pulp). Pulp tissue (5 g) was extracted with 20 ml of chloroform-methanol solvent (2:1, v/v). Data are representative of two additional trials. A similar increase in lipofuscin was also observed in the peel tissue. Excitation wavelength was 360 nm and emission wavelength was 440 nm.



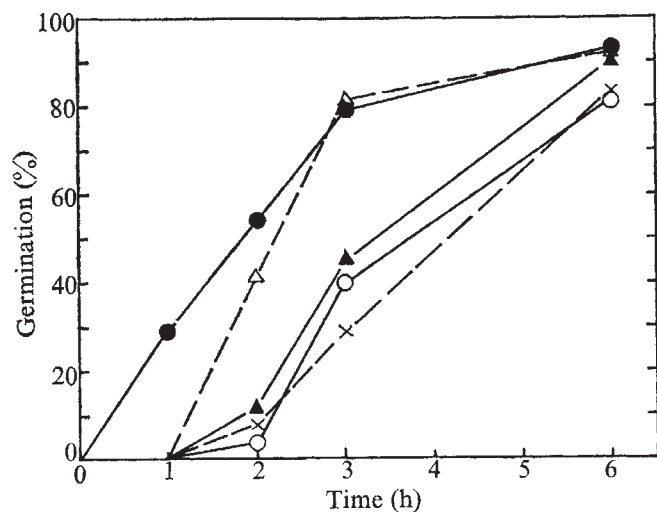


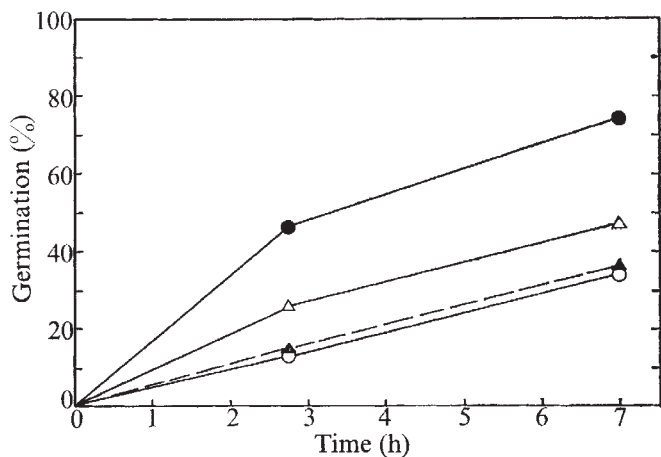
Fig. 1 Percentage germination of pollen, one week old, humidified, with con A in the germination medium. ●, 1 mg ml^{-1} con A; △, $100 \text{ } \mu\text{g ml}^{-1}$ con A; ▲, $10 \text{ } \mu\text{g ml}^{-1}$ con A; ×, $1 \text{ } \mu\text{g ml}^{-1}$ con A; ○, control.

second hour. At concentrations of $10 \text{ } \mu\text{g ml}^{-1}$ con A, the germination rates were only slightly higher than the control rates and at $1 \text{ } \mu\text{g ml}^{-1}$ con A, they were slightly lower than the controls. The total percentage of pollen which germinated approached the same value for the control after about 6 h as for all the con A concentrations tested. With the addition of $0.1 \text{ M } \alpha$ -methyl mannoside, a competitive inhibitor of con A binding, the stimulatory effect of con A was reduced by 20–50%.

In older pollen, which had been stored for seven weeks, the lag period was shortened by about 2 h and the maximum percentage germination attained was doubled when con A was added to the germination medium (Fig. 2).

The stimulatory effect of PHA was greater than that of con A (Fig. 3). The greatest number of pollen grains germinated during the first hour with PHA (1 mg ml^{-1}), during the second hour with con A (1 mg ml^{-1}) and no sooner than the fourth hour in the control. Comparable experiments which ran for a longer time (up to 20 h) showed that the maximum percentage of grains eventually germinating in the control was similar to that germinating in either PHA or con A. Pollen in $100 \text{ } \mu\text{g ml}^{-1}$ PHA germinated slightly faster than the control, while the difference between germination rates in $10 \text{ } \mu\text{g ml}^{-1}$ PHA and in the control was not significant.

Fig. 2 Percentage germination of pollen, seven weeks old, humidified, with con A added to the germination medium. ●, $250 \text{ } \mu\text{g ml}^{-1}$ con A; △, $100 \text{ } \mu\text{g ml}^{-1}$ con A; ▲, $2.5 \text{ } \mu\text{g ml}^{-1}$ con A; ○, control.



Since lectins did not increase the maximum percentage of grains which eventually germinate, their action may be similar to the events which occur spontaneously over a longer period. The stimulation of germination by lectins which are known to bind to cell surfaces of red blood cells, fibroblasts, lymphocytes and spermatozoa from a range of animals³ suggests that primary control of the germination process may occur at the plasma membrane. Pollen germination is affected by sugars and ions, especially calcium, which are taken up from the external medium either *in vivo* or *in vitro*^{8,9}. An alteration of the permeability properties of the plasma membrane by bound lectins could lead to greater uptake of water or nutrients for germination. Ion fluxes involving calcium have been described around germinating lily pollen and localised influxes of cations have been postulated to influence pollen tube growth¹⁰. Substances which bind con A have been found in the walls of pollen grains; however, they can be readily leached out of the walls and do not seem to remain bound to the cell wall or plasma membrane¹¹.

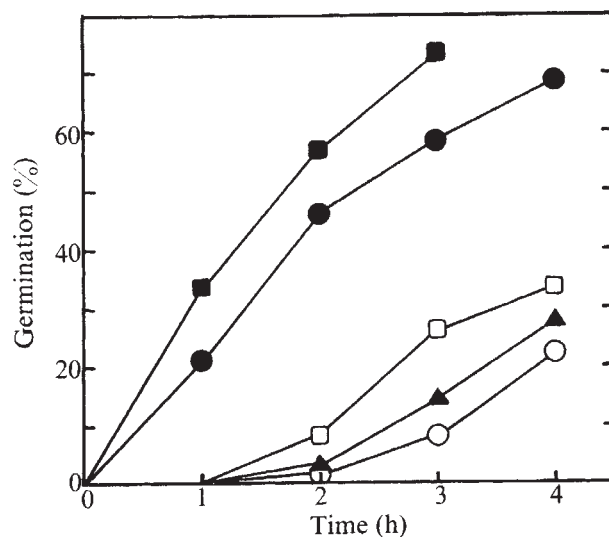


Fig. 3 Percentage germination of pollen, one week old, un-humidified, with PHA added to the germination medium. One sample was given con A. ●, 1 mg ml^{-1} con A; ■, 1 mg ml^{-1} PHA; □, $100 \text{ } \mu\text{g ml}^{-1}$ PHA; ▲, $10 \text{ } \mu\text{g ml}^{-1}$ PHA; ○, control.

Although con A and PHA stimulate the uptake of ^3H -thymidine by lymphocytes^{3,12} it seems unlikely that they exert their effect on the synthetic activities of germinating pollen. Neither DNA nor RNA synthesis is required for pollen tube initiation¹³.

The stimulation of pollen germination by con A and PHA as manifested by a reduction in the length of the lag period is similar to the stimulatory effect of the stigmatic exudate on pollen germination¹⁴. It is therefore possible that the events mediated by lectins *in vitro* may also occur during normal germination *in vivo*.

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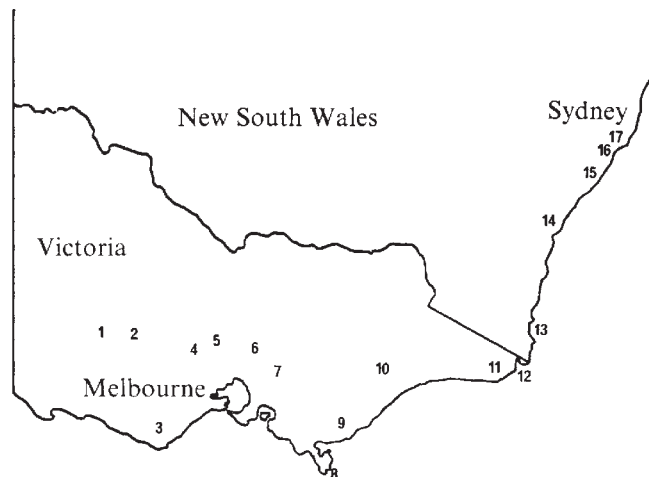


Fig. 1 Map of Victoria and south-eastern New South Wales indicating continental islands where endemic *Drosophila* spp. have been found.

Adaptive radiation in the subgenus *Scaptodrosophila* of Australian *Drosophila*

UNTIL a recent survey, based on the two major museum collections of Australia¹, the Australian *Drosophila* fauna was poorly known and few endemic species had been described. This survey covered 81 species including 40 described as new. The world total of *Drosophila* species is known to exceed 1,250 (ref. 2); the Australian fauna thus represents about 6% of this total. By comparison with the world *Drosophila* fauna, the Australian total contains a disproportionately large number of species (45 out of 81) in the subgenus *Scaptodrosophila*, most of them endemic although a few are also known from neighbouring South-east Asian and/or Pacific regions. The Australian *Drosophila* fauna is therefore unique in that nowhere else have members of the subgenus *Scaptodrosophila* so dominated the *Drosophila* fauna of a large region.

The three other major subgenera are poorly represented. The eleven known Australian *Hirtodrosophila* species are all endemic to Australia, but in the subgenera *Drosophila* and *Sophophora*, each of which contains more than 500 species worldwide³, four species only of *Sophophora* are endemic to Australia; these subgenera are represented in Australia principally by the cosmopolitan species, although a few of the South-east Asian/New Guinean species extend into northern Australia.

Of the *Scaptodrosophila* species, only three are endemic to south-western Australia; a further two occur in both east and west, and the remainder have southern and/or northern distributions in the eastern part of the continent only¹. Laboratory culture of most species has proved very difficult, complicated by the almost complete lack of information on ecology and life histories; there are some indications that the larvae of at least some species may be leaf miners pupating in soil. Equally, phylogenetic lineages within the subgenus are substantially unknown¹. The Australian *Scaptodrosophila* radiation does not, of course, compare with the famous speciation in the subgenus *Drosophila* in Hawaii⁴, in which small area more than 350 species have already been described—about four times the total number of known Australian species in the genus *Drosophila* and seven times the number of known Australian *Scaptodrosophila*; but the Hawaiian proliferation is a special case unmatched in any other comparably small region of the world.

The major *Scaptodrosophila* radiation in Australia has thus occurred in the eastern part of the continent, and within this region many of the species seem to be restricted to the southern part of the country (especially Victoria and New South Wales). One of the more common species is *D. inornata* Malloch, which is frequently closely associated with tree ferns (*Dicksonia* spp.)⁵. The most favourable habitats for *Drosophila* species in south-eastern Australia seem to be wet sclerophyll/rain forest areas characterised by tree ferns and various other ferns. All habitats, including a few sheltered habitats without tree ferns, have permanent water, and often a deep layer of rotting leaf litter⁶. Most of the *Scaptodrosophila* species are not attracted to baits and so far can only be collected by intensive sweeping.

Extensive spring and summer collections have recently been made in habitats of the above type in Victoria and the east coast of southern New South Wales (Fig. 1). More than 2,000 *Scaptodrosophila* representing at least twelve species, at least two of them new, were collected in damp habitats. Locality records of the museum collections¹ reveal the likelihood that most of the remaining known species are confined to damp habitats. It seems likely in view of the number of sites at which collections are still to be made that further species await discovery; in any case species once regarded as extremely rare are now known to be relatively common and widespread.

The damp habitats most favourable to *Scaptodrosophila* species are normally isolated from one another, often by considerable distances. Localities of major habitats sampled so far are indicated in Fig. 1. The temperature/humidity requirements of the flies are such that migration among many of the habitats in summer, especially in Victoria, must be impossible⁶. This is because Victoria has a hot dry summer during which the habitats are damp, cool refuges, offering temperature–humidity conditions compatible with survival of *Drosophila* species, but outside these refuges the environmental stress would be excessive. (Similar environmental constraints occur for the cosmopolitan species⁷.) There is, therefore, the possibility of studying the evolutionary biology of *Scaptodrosophila* species in essentially continental islands which have probably been separated for at least 6,000 yr, that is after the end of an era of high precipitation, high temperature and high rainfall⁸.

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Autonomous pigment movement in the radial pupil of locust ocelli

ALTHOUGH pigment movement is important for modulating luminous intensity on the invertebrate retina, its mechanisms and control cannot be investigated easily since the cells in question are largely inaccessible to experimental interference and often observable only by electron microscopy. The preparation described here is directly observable and is also potentially amenable to manipulation.

The ocellus of the mature adult locust is a simple lens eye comprising a lens up to 500 μm diameter overlying a transparent vitreous layer and retina, behind which is a neuropile and ocellar nerve¹. The whole cup-shaped structure is invested by a densely pigmented epithelial layer continuous with the epidermal cells outside the ocellus and a ring of specialised epidermal cells lying between the vitreous layer and the lens. The cells of this ring adjacent to the lens are clear corneagenous cells. The more proximal of these cells contain numerous reddish-brown pigment granules which in *Schistocerca* are confined to the periphery of the ocellus and thought to be stationary¹. My observations of an Indonesian acridid, *Valanga nigricornis* and an Australian species, *Austracorus guttulosa* indicate that this pigment is moveable in some acridids.

Light-adapted ocelli show a distinct pupil lying in the middle of the eye, on dark adaptation the pupil expands so that the eye presents a pure white fundus with only a margin of pigment visible (Fig. 1a). Pigment movement has no apparent circadian rhythm and is controlled only by light falling on that ocellus, illumination of the compound eyes or other ocelli being ineffective. The fully contracted pupil may be a circular aperture or a vertical slit. The Indonesian species examined showed circular pupils in lateral and median ocelli whereas *Austracorus* showed slits on the lateral ocelli and a round pupil on the median. The degree of pigment movement varies widely between individuals and may differ considerably between the right and left ocelli of a single animal. Close inspection of ocelli under a dissecting microscope revealed an optical discontinuity in the centre of the lens which proved, in the scanning electron microscope, to be the result of a circular or oval ridge on the back surface (Fig. 1b). While pigment in the extreme light-adapted condition of some ocelli did not extend as far as this ridge, pigment was never found to intrude within this circle, and in many cases the pupillary margin was defined by this ridge (for example, Fig. 1a). Light and transmission electron microscopy show the central region of the lens to be free from pigment and corneagenous cells, the vitreous cells here abutting the lens directly. The crest of the ridge is also punctuated with holes of about 2 μm diameter, which in life are presumably occupied by processes from the vitreous cells to form a stockade delineating the inner margin of the pigment cells.

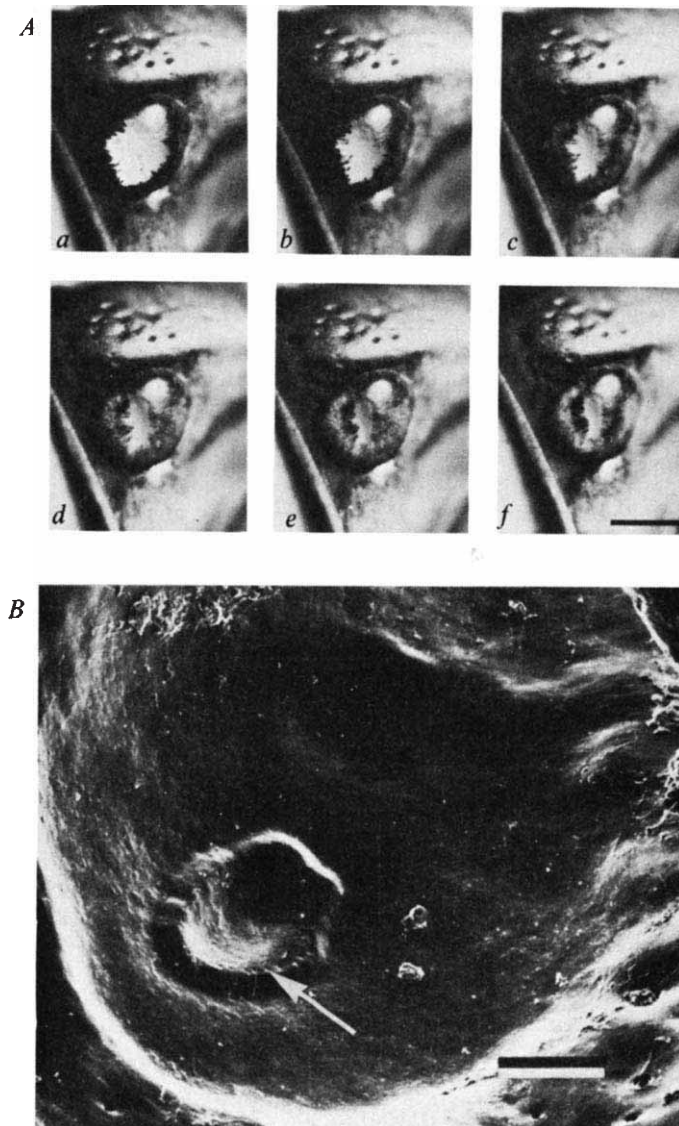
The efficiency of the pupil as a mechanism for reducing the light intensity at the retina cannot be calculated readily unless it can be shown that the field of view of the ocellus is not significantly influenced by pupil diameter, direct measurements of field size show that this condition is satisfied (unpublished observations). As Fig. 2a shows, the area reduction in the pupil is of the same order of magnitude as the light reflex in the human eye², the time course on the other hand is orders of magnitude slower. The response-intensity function for pigment cells has been quantified by observation of pupil diameter through a Leitz incident illumination microscope used with Ultrapak objectives. Incident light passing through a narrow-band interference filter (546 nm peak) and attenuated with neutral density filters was allowed to fall on an ocellus of an intact but immobilised animal. Measurement of pupil diameter was made in red light (628 nm) after 90 min adaptation to the test intensity. The dynamic range of pigment movement extends for only 2.5 log units, by visual standards a narrow range (Fig.

2b). In comparable conditions the human pupil has a dynamic range of 7 log units (ref. 2).

In view of this narrow dynamic range the control mechanism for pigment movement is particularly interesting. Some simple experiments were therefore carried out to explore the obvious proposition that pigment movement requires the neural elements of the ocellus.

Once the head capsule is opened, gentle pulling on the ocellar nerve will detach the vitreous from the pigment ring distal to it. The lens and its adhering pigment iris may then be isolated and observed *in vitro*. Unfortunately the pigment cells do not survive well in the usual locust Ringer solutions^{3,4}. Immersion in silicone oil is less injurious, and in favourable preparations reversible pigment movement in response to light can be elicited for several hours after excision. The trauma of surgery, however, is a frequent cause of failure. A much more reliable preparation is obtained by allowing the pigment to remain in the haemolymph in the absence of the neural elements of the

Fig. 1 A, Contraction of the pupil in the right lateral ocellus of *Austracorus* after the onset of an intense white light. The fully contracted pupil is clearly a vertical slit in these ocelli, the optical discontinuity caused by the ridge on the back of the lens may just be discerned in (b) and (c). Times after stimulus onset were: a, 0 min; b, 3 min; c, 6 min; d, 9 min; e, 15 min; f, 40 min. The bar represents 500 μm . B, Scanning electron micrograph of the back surface of a lateral ocellus from an Indonesian acridid. The whole lens is a shallow concavity in the centre of which is a pronounced circular ridge. The crest of the ridge is punctuated with 2- μm diameter holes, indicated by the arrow, the bar represents 100 μm .



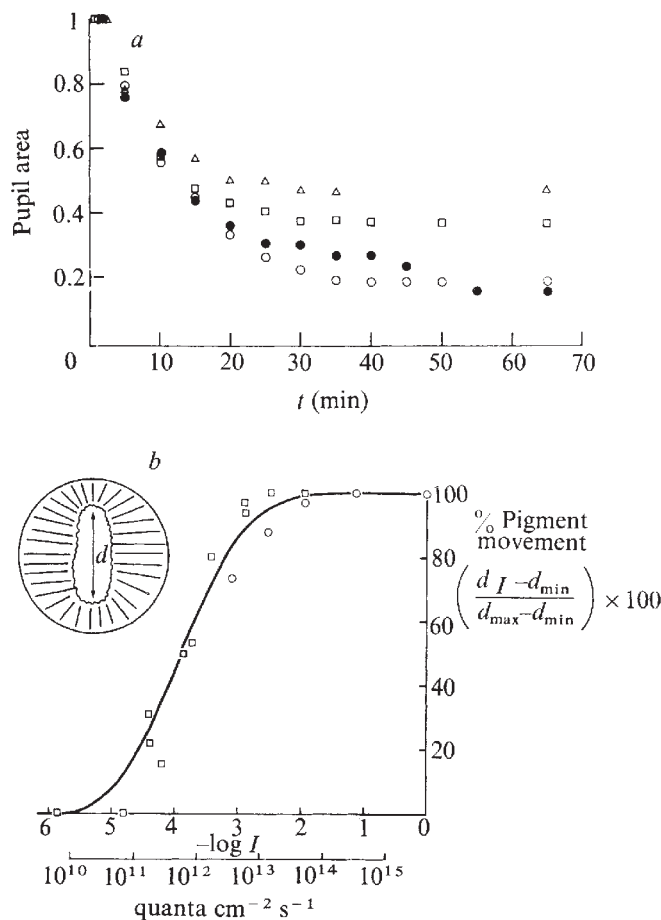


Fig. 2 *a*, Reduction in pupil area as a function of time after intense stimulus onset. The results from four lateral ocelli of *Valanga* normalised relative to their initial (dark adapted) pupil areas, maximum apparent pupil area was about 0.25 mm². Making reasonable assumptions about the positions of the principal planes of the thick lens in these ocelli the real pupil is unlikely to be more than 15% smaller than the apparent magnified pupil. *b*, Pigment movement as a function of intensity of monochromatic green light for a lateral ocellus of *Austracorus*. □, Long axis of the pupil; ○, short axis. Quanta $\text{cm}^{-2} \text{s}^{-1}$ were determined by substituting a calibrated PINIO diode for the ocellus. Since the incident illumination was not perpendicular to either ocellus or detector but was a hollow cone of light with the object at the apex these figures are only approximations.

ocellus. A small hole in the exoskeleton dorsal to an ocellus allows the introduction of a tungsten wire hook with which the ocellar bulb may be extracted from the head capsule. A cut then serves to remove the ocellus and a length of ocellar nerve. If the hole is revealed the pigment remaining attached to the lens is viable for several days, responding to light as it did before surgery.

From the simple experiments reported here it is concluded that pigment movement in the ocellar iris is truly autonomous, being controlled by neither neural nor hormonal mechanisms but by processes intrinsic to the pigment cells. Although nothing is known about the method of transduction of light by these pigment cells, the recent reports of transduction by pigment grains within *Aplysia* central neurones suggest an obvious parallel^{5,6}. The significance of the pupil in the functioning of the ocellus is presumably as a mechanism for modulating the intensity of light at the retina, for the other two possibilities—increased depth of focus⁷ and reduction in field of view (unpublished observations)—have been eliminated. Although the maximum reduction of intensity achieved by the pupil is a meagre 0.7 log units it is likely that, as in the case of the human pupil⁸, even a modest effect is worthwhile.

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Effects of hyperthermia on rat embryos in culture

TEMPORARY elevation of the body temperature, resulting from climatic influences or infection, occurs in many mammals. Such hyperthermia is often harmless, but in the pregnant female it may be teratogenic, with particularly damaging effects on the brain of the developing embryo. The studies that have been made on experimentally induced hyperthermia and teratogenesis in animals are reviewed in ref. 1 and suggest that hyperthermia may also be teratogenic in man. The methods used to raise the temperature in experimental animals have included incubation of the whole animal², injection of foreign protein³, short-wave diathermy⁴, immersion of the uterus in heated saline^{5,6}, and microwave radiation of the uterus⁷. In most of these experiments the period of exposure to high temperature (often only 40–60 min) was limited by the tolerance of the animal and the need to avoid excessive resorption of the embryos. The temperature experienced by the embryos usually varied during treatment and could not be controlled precisely.

When the embryos are explanted and grown in culture they may be exposed to raised temperatures for longer and in closely specified conditions. It also becomes possible to distinguish direct effects of hyperthermia from those that may result indirectly from changes in the maternal metabolism. We report here some results obtained from a study on rat embryos explanted during early organogenesis (a stage of development known to be particularly sensitive to teratogenic agents) and grown in culture in various conditions of hyperthermia.

Rat embryos of CFHB strain were explanted on the tenth day of pregnancy and grown in culture for 46–47 h in rotating glass-stoppered bottles by the method of New *et al.*⁸. The culture medium was pure rat serum, containing 50 $\mu\text{g ml}^{-1}$ streptomycin, equilibrated with a gas mixture of 5% O₂, 5% CO₂, 90% N₂ which was changed to 20% O₂, 5% CO₂, 75% N₂ after the first 24–27 h of culture. The culture bottles were rotated in purpose-built incubators with an overall temperature accuracy of $\pm 0.2^\circ\text{C}$. In earlier experiments, the control incubator was set at 37°C , but this was later raised to 38°C to conform more closely with measurements of deep rectal temperature of rats at mid-pregnancy. No difference was found in development at 37°C or at 38°C .

At explantation, the embryos were 'egg cylinders', 1–2 mm long. In most, the earliest stages of neural fold development were visible but no further organogenesis had occurred.

Table 1 Development of embryos grown in culture during the temperature schedules shown in Fig. 1

		Total no. of embryos	Blood circ.	No. of embryos showing: Turning completed	Fused allan.	Normal organ.	Final protein (μg mean $\pm$ s.e.)
Series A	a	27	23	18	22	27	130 $\pm$ 12
	b	27	16	20	17	13	127 $\pm$ 10
	c	27	0	3	3	2	52 $\pm$ 6
Series B	a	12	9	11	12	12	127 $\pm$ 10
	b	12	1	4	12	9	65 $\pm$ 5
	c	12	0	0	12	0	43 $\pm$ 3
Series C	a	18	11	14	14	15	176 $\pm$ 9
	b	18	3	11	8	5	134 $\pm$ 9
	c	18	15	17	13	15	146 $\pm$ 10
Series D	a	41	40	38	37	39	121 $\pm$ 6
	b	41	32	25	34	31	101 $\pm$ 4
	c	41	33	21	32	25	90 $\pm$ 4

Blood circ., Indicates the number of explants which showed a normal vigorous blood circulation in both embryo and yolk sac at the end of the culture period.

Turning completed., Number of embryos which had completed the rotation from the early dorsally-concave curvature to the more characteristic foetal position with the ventral surface concave.

Fused allan., Indicates that the allantois had extended from the hind end of the embryo and fused normally with the chorion.

Normal organ., Indicates that no abnormalities of organogenesis could be detected.

After examination, most of the embryos (without the embryonic membranes) were digested in caustic soda and assessed for total protein content by a colorimetric method⁹.

At the end of the culture period, most of the embryos incubated at control temperature had formed about 23–26 somites with brain and spinal cord, optic and otic vesicles, anterior limb buds and a well-developed heart and blood circulation (Fig. 2). A yolk sac, amnion and chorioallantois were also present.

Four series of experiments were made, with temperature schedules as shown in Fig. 1. In each series, one group of embryos (a) was cultured at control temperature and the other two (b, c) were exposed to raised temperature for some time. To ensure that the three groups were comparable, each litter of embryos was divided equally between them. The results are summarised in Table 1.

In series A (81 embryos) a comparison was made of the development of embryos exposed to control temperature (37–38 °C), to 40 °C, and to 41 °C throughout the culture period. At 40 °C, overall growth was similar to that of the controls (Fig. 2) although a smaller proportion of these embryos had a good blood circulation at the end of culture, and about half showed small developmental abnormalities, including a few with slight microcephaly. In contrast, the embryos incubated at 41 °C were very severely affected. Final protein content was very significantly less than that of embryos incubated at either 37–38 °C or 40 °C ($P < 0.001$). Somite formation was so poor at 41 °C that somite counts could not be made. More than half the embryos in this group were microcephalic and almost as

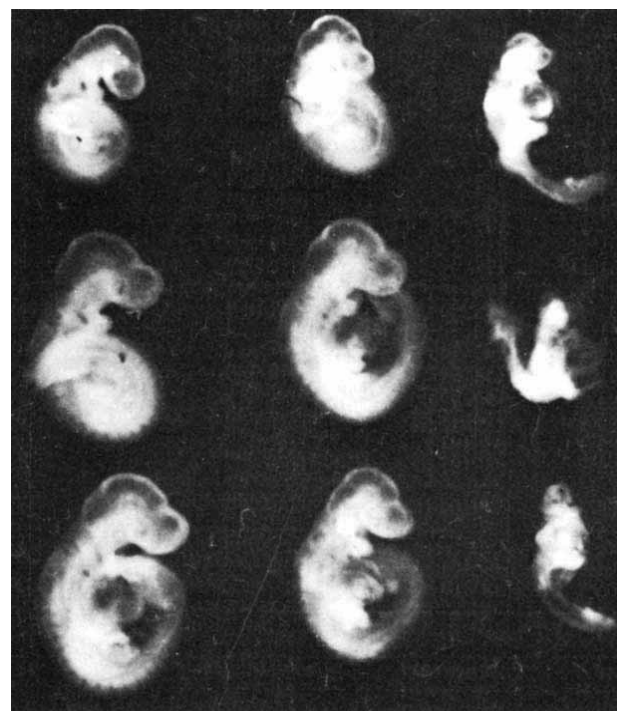
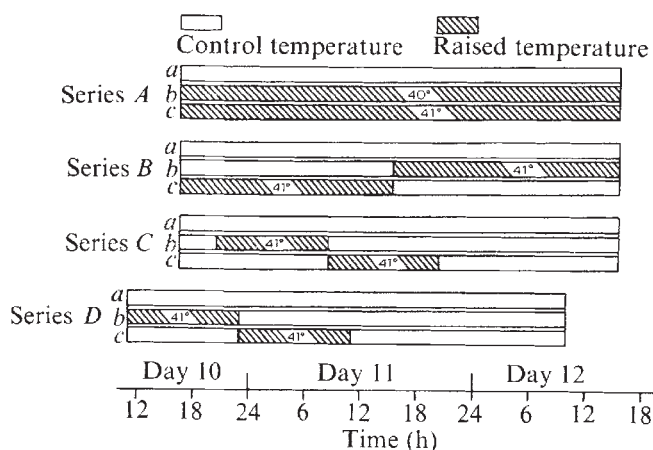


Fig. 2 Nine embryos from the same rat, explanted at early head-fold stage and grown in culture for 46 h (series A). Incubated at 38 °C (left), 40 °C (centre) and 41 °C (right). (The embryonic membranes have been removed.)

Fig. 1 Schedules of heat treatment applied to rat embryos in culture.



many had enlarged hearts and oedema of the pericardium; few had begun to turn, and in 6 of the 27, the posterior part of the trunk was sharply bent back on the anterior, with fusion of the anterior and posterior neural folds.

In series B (36 embryos), the duration of exposure to 41 °C was reduced to half the 46-h culture period, but this was enough to produce many abnormalities. The embryos incubated at 41 °C for the first 23 h had a very low protein content and were much smaller than the controls. The somites were badly developed. None of these embryos had turned, and none had a blood circulation at the end of culture. Half were microcephalic and nearly as many showed pericardial oedema. The embryos incubated at 41 °C for the second 23 h were better formed, though a few had turned and only one had a normal blood circulation. They were also very small, with a protein content significantly lower than that of the controls ($P < 0.001$).

though significantly higher than that of the embryos exposed to 41 °C for the first half of culture ($P < 0.002$).

In series C (54 embryos) and D (123 embryos), exposure to 41 °C for various 12-h periods produced relatively few gross malformations, but the protein content was always significantly lower than that of the controls. The most severely affected embryos were those in group b of C and in group c of D. Both were exposed to 41 °C during the first few hours of day 11, which may indicate that this is an exceptionally sensitive period. Common abnormalities were microcephaly and pericardial oedema. Blood circulation, rotation of the embryo, and fusion of allantois and chorion were also affected.

A notable feature of these results is the very small temperature rise needed to produce abnormalities of development. Some defects were apparent when the temperature was raised only 2 degrees above the normal for rats (that is, to 40 °C) and gross abnormalities were found in nearly all the embryos when the temperature was raised to 41 °C throughout the culture period. Such small rises are well within the range commonly experienced during fever and suggest that studies of this kind on the embryos of laboratory animals in culture may be relevant to problems of hyperthermia and teratogenesis in man. The frequent appearance of defects of the head agrees with the findings of others^{2,5,6} on hyperthermia applied to embryos *in vivo* at this stage of development. Finally, it may be noted that the present study has demonstrated direct effects of hyperthermia on the development of mammalian embryos isolated from the maternal metabolism.

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Enzyme variability in the protochordate amphioxus

COMPARING protein polymorphisms in 24 species of invertebrates and 22 species of vertebrates, Selander and Kaufman¹ observed greater genetic variability on average in the invertebrate than in the vertebrate by three criteria: number of polymorphic loci (25–50% in invertebrates compared with 10–20% in vertebrates); mean heterozygosity per locus per individual (15% compared with 6%); and, average number of alleles per polymorphic locus (three compared with two). I have found that amphioxus, a fish-like protochordate, combining vertebrate characters (such as dorsal neural tube throughout life) and invertebrate characters (such as solenocytic protonephridia) resembles, in its enzyme variability, invertebrates rather than vertebrates.

I have done electrophoresis of extracts of 44 specimens of an Australian species of amphioxus, *Branchiostoma moretonensis*², collected in Moreton Bay, Queensland and flown to Adelaide. All individuals were surveyed both in starch gel and in acrylamide gel. Most variation was observed in both systems, although to identify isoalleles glucose-6-phosphate dehydrogenase had to be run in parallel in both systems. Bands that had been thought to be two distinct loci coding for the

set of rapidly migrating glucose-6-phosphate dehydrogenases in *Branchiostoma lanceolatum*³ represent variants with identical electrophoretic mobility of the major zone but non-identical mobility of the satellite zone. The enzymes listed in Table 1 were localised, after electrophoresis, by conventional methods^{4,5}.

NADP malate dehydrogenase, 6-phosphogluconate dehydrogenase, glutamate dehydrogenase and alcohol dehydrogenase, usually readily detectable after electrophoresis of extracts of tissues from most animals, could not be localised after electrophoresis of amphioxus extracts³. For some extracts extremely faint zones were observed after 24 h of staining for NADP isocitrate dehydrogenase and for L-lactate dehydrogenase; such low activity may represent reaction on contaminants in the staining reagents and, in any case, is likely to be unreliable in scoring for variation.

Table 1 shows that amphioxus resembles Selander and Kaufman's¹ average invertebrate rather than their average vertebrate in the amount of enzyme variability. For 10 out of 14 enzyme loci there is clear genetically interpretable⁴ variation, with an average of 4.2 alleles per locus. The average amphioxus is heterozygous at 24% of these enzyme loci. Using Avise and Selander's⁶ definition that a locus is polymorphic only when the commonest variant is present in an allelic frequency of 0.95 or less, nine of the 10 loci with genetically interpretable variation are polymorphic. It can be calculated that for a sample of 44 individuals, representing a sample of 88 genes for each diploid autosomal locus, the chances of detecting variation at this 0.95 cutoff point is close to 99%— $(0.95)^{88} = 0.011$.

One unusual observation is that there is a large deficit of homozygotes for the phosphoglucomutase polymorphism: 22 homozygotes were observed compared with 9.74 expected. A binomial test⁷ shows that the probability of such an extreme deviation is less than 0.001. Ayala *et al.*⁸ observed a statistically significant deficit of heterozygotes for phosphoglucomutase in the clam *Tridacna maxima* but explained this observation as "our scoring is biased against heterozygous genotypes" (page 185) as a result of "allozymes with little difference in migration" (page 186).

There is no overlap between any of the six variants of phosphoglucomutase in amphioxus; thus, the very highly statistically significant deficit of heterozygotes cannot be the result of problems in electrophoretic resolution. In the survey of amphioxus no other polymorphic protein had a statistically significant deficit, or excess, of heterozygotes.

The data on amphioxus are significant from three other aspects.

First, there is partial association of variants of glucose-6-phosphate dehydrogenase and phosphoglucomutase. Individuals with the electrophoretically faster variants of phosphoglucomutase tended to have a somewhat greater frequency of the electrophoretically slower variants of glucose-6-phosphate dehydrogenase and vice versa (Table 2). The probability of such a partial association occurring by chance can be estimated by treating the data in Table 2 as a 2 × 2 contingency table and, after correcting for continuity, calculating the χ^2 statistic⁹; the χ^2 is 6.71 with one degree of freedom, significant at the 0.01 level. No other association of variants for different enzymes was observed. As phosphoglucomutase and glucose-6-phosphate dehydrogenase are adjacent enzymes in connecting glycolysis and the pentose pathways, this epistatic interaction may be biochemically significant.

Second, these results demonstrate variability in a 'primitive' animal. Although amphioxus has some characteristics which probably represent secondary specialisation, there is no doubt that it is a primitive form, resembling a hypothetical pre-vertebrate so well that "if amphioxus had not been discovered it would have to have been invented" (page 388 of ref. 9). According to J. Z. Young¹⁰ (page 46) "amphioxus shows us approximately the condition of the early fish-like chordates, living in the Silurian some 400 million years ago, and . . . has undergone relatively little change in all the time since". My

Table 1 Enzyme polymorphism in amphioxus

Enzyme (EC number)	No. of alleles	Gene frequencies*	Hetero- zygosity (%)
Group I enzymes (components of energy yielding and major biosynthetic pathways ¹²)			
Hexokinase (2.7.1.1)	3	0.02; 0.83; 0.15	26
Phosphoglucumutase (2.7.5.1)	6	0.04; 0.22; 0.28; 0.15; 0.26; 0.04	50
Glucose-6-phosphate dehydrogenase (1.1.1.49)	8	0.11; 0.16; 0.27; 0.02; 0.24; 0.15; 0.01; 0.03	68
'Fast'	Variable		
'Slow'			
Glucose-phosphate isomerase (5.3.1.9)	3	0.04; 0.92; 0.04	14
NAD diaphorase (1.6.-.-)	2	0.01; 0.99	
NAD-dependent L-malate dehydrogenase (1.1.1.37)	Monomorphic		
'Fast'			
'Slow major'			
	2	0.07; 0.93	6
Group II enzymes (relatively nonspecific: esterases, peptidases and so on ¹²)			
Tetrazolium oxidase	Monomorphic		
α -Naphthyl acetate esterases (3.1.-.-)	8	0.08; 0.19; 0.23; 0.16; 0.19; 0.08; 0.03; 0.03	84
'Fast'			
'Middle'	2	0.08; 0.92	
'Slow'			
Phe-leu peptidases (3.4.-.-)	Monomorphic		
'Fast'			
'Slow'			
	4	0.04; 0.81; 0.13; 0.02	29
	2	0.62; 0.38	41

* Listed in order of electrophoretic mobility, pH 8.0 starch gel; most anodal variant first.

findings with amphioxus resemble those of Selander *et al.*¹¹, who observed a moderately large amount of variation in a survey of 16 enzymes in the xiphosuran *Limulus polyphemus*, a phylogenetic relict of at least 200 Myr. The living representatives of these two bradytelic lines are no less polymorphic, at

in Table 1 except for 'slow' glucose-6-phosphate dehydrogenase. Several enzymes in the energy-yielding and major biosynthetic pathways are large molecules, often composed of several subunits. While it is reasonable to expect that such enzymes may be more conservative in their electrophoretic variability,

Table 2 Partial association of variants of phosphoglucumutase and glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase	Phosphoglucumutase	
	The two electrophoretically faster variants (A and B)	The four electrophoretically slower variants (C, D, E and F)
The four electrophoretically faster variants (A, B, C and C')	12 (15.5)*	53 (39)*
The four electrophoretically slower variants (D, E, E' and F)	19 (15.5)*	25 (39)*

* The numbers in parentheses are the expected values for each category if the numbers were distributed at random.

least at the enzyme level, than representatives of rapidly evolving lineages.

Third, there is the variability in enzymes involved in energy production and major biosynthetic pathways, compared with more peripheral enzymes of often lower substrate specificity. Enzymes active in energy metabolism, "group I", have less electrophoretic variation than the so-called "nonspecific enzymes", "group II", in which have been placed esterases, phosphatases, peptidases, amylases, alcohol dehydrogenases and aldehyde oxidase^{12,13}. Amphioxus has less heterozygosity for the group I enzymes (Table 1), but the difference is not statistically significant: 77 heterozygotes out of 352 opportunities for group I enzymes compared with 71 heterozygotes out of 264 opportunities for group II enzymes— $\chi^2 = 1.82$ (1 d.f.). I found sharp electrophoretic resolution for all enzymes

it is also possible that some of the apparent lack of polymorphism reported in the literature is the result of limitations in electrophoretic technique where large multi-subunit enzymes often give less sharp resolution than small single-subunit proteins.

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Non-selective isolation, stability and longevity of hybrids between normal human somatic cells

FIFTEEN years after the first somatic cell hybridisation¹, there are still no reports of the successful isolation of proliferating populations of pure synkaryons between strains of euploid mammalian cells. Migeon *et al.*² fused suitably marked human fibroblasts and lymphocytes using a selective medium, but they were unable to isolate further and characterise the presumptive hybrid subpopulation. No one, to our knowledge, has been able to obtain synkaryons between euploid cells without the use of biochemical selective techniques. Such an achievement would be highly significant, since it would facilitate mating between any pair of euploid somatic cells for which there are few selective², but many³ differentiating markers.

In the experiments summarised in Table 1, we fused fibroblast cultures from pairs of normal male human donors, one with the A, the other with the B form of X-linked glucose-6-phosphate dehydrogenase (G6PD). The data of Table 2 were derived from fusions among cells of a skin culture from a

Table 1 Summary of cytogenetic and electrophoretic classification of clonal isolates from 8 separate fusion experiments involving 5 different G6PD_A and G6PD_B strains

Type of fusion:	Total clones screened/isolated (3,203/108*)			
G6PD _A × G6PD _B (male strains)	G6PD A	G6PD B	G6PD A + B	G6PD Heteropolymer
100% Diploid	10	15	5	
> 90% Diploid	3	1	1	
Diploid + tetraploid		24	12	2
> 90% Tetraploid		7		1
100% Tetraploid	3	15		3

* 6 not tested.

Fibroblast cultures were established from foreskins or skin biopsies and grown according to techniques described previously⁴. For fusion, 0.5×10^6 mitotic or interphase cells of each of the G6PD_A and G6PD_B strains, and 1×10^6 cells in the case of the G6PD_{AB} heterozygote, were suspended in 0.5 ml of chorioallantoic fluid⁵ containing inactivated Sendai virus and kept on ice. After 20 min, the virus cell suspensions were transferred to a 37 °C water bath and incubated for 60 min. The preparations were then diluted with 10 ml of complete media, centrifuged, resuspended, and plated into 2-4 60-mm dishes at a density of 9,000 cells per cm². Twenty-four hours later, one of the dishes was trypsinised and dilute-plated at densities of 6-8 cells per cm² into 100-mm Petri dishes. A typical experiment involved the preparation of 10 such dishes, which received two feedings before they were screened for the presence of tetraploid clones at days 9-11 after plating. Tentative identification of tetraploid clones was based on visual comparison (Nikon inverted microscope, model MS) of the sizes of the equatorial plates⁶ of metaphases of the living clones contained within a 100-mm dish. Isolation of all clones judged to be tetraploid was by a steel cylinder technique⁷. All isolates were transferred to 35-mm Petri dishes and further subdivided after 3-5 d. One dish each was used for cytogenetics, electrophoresis and further passage. For cytogenetics, an *in situ* technique⁸ was used which provides chromosome spreads on 2 cm² coverslips. An average of 50 spreads was counted per preparation. Electrophoresis was conducted on cellulose acetate plates².

female heterozygous for G6PD_A and G6PD_B, some of her cells being of the A type and others of the B type. In theory, one-third of the tetraploid clones emerging from dilute platings of approximately equal cell mixtures of G6PD_A and G6PD_B types should be identifiable as hybrids, provided that there is no preferential fusion between like cells, no precipitous chromosomal loss or diploidisation during the initial period of clonal growth, no significant tetraploid cell population due to causes other than cell fusion, and no selective growth for hybrids, selfed tetraploids or parentals. Our results show that hybrid isolates were, in fact, obtained, even though at yields substantially below expectation. Among the mixoploid isolates listed in Tables 1 and 2, there was a total of five cultures with a distinct heteropolymer component in addition to the A and B band, but the distribution of presumptive subunits deviated from the typical hybrid (1:2:1) pattern.

The success of the procedure we describe seems to be dependent on sufficient growth potential of each of the parental strains, effective heterokaryon formation and ability to identify tetraploid colonies. We found that our previously described, relatively laborious quantitative method of ploidy determination⁶ could be simplified by visual comparison of metaphases of suspected tetraploid clones with those of control diploids (the predominating type) in the same dish. With some experience, it is possible to screen and classify 200-300 colonies within one hour. We believe that most of our mixoploid isolates arose not from erroneous identification, but from contamination with diploid cells during the plating and/or isolation procedures, which obviously can be minimised by plating at lower densities and by earlier transfers of presumptive tetraploid clones (before contact between colonies). We have

Fig. 1 Growth rates and longevity of six hybrid cultures. Starting at passage 5 after isolation, 5×10^4 cells were seeded into 75 mm² plastic culture flasks and passaged at weekly intervals until there were less than input cells after one week's incubation. Estimates of the longevity of these cultures were based on haemocytometer cell counts. These counts were converted into averages of population doublings per day as a measure of growth kinetics, and into cumulative population doublings (numbers in parentheses next to the strain identification number) as a measure of total growth potential⁴.

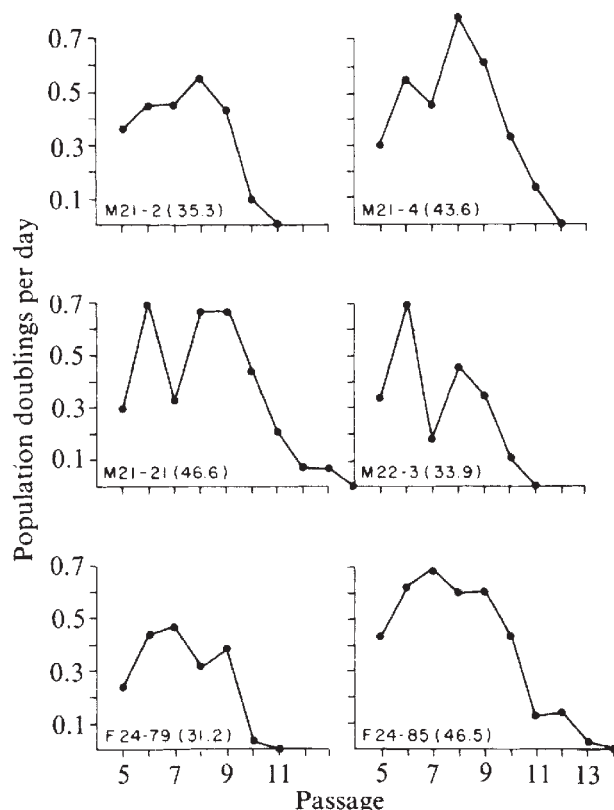
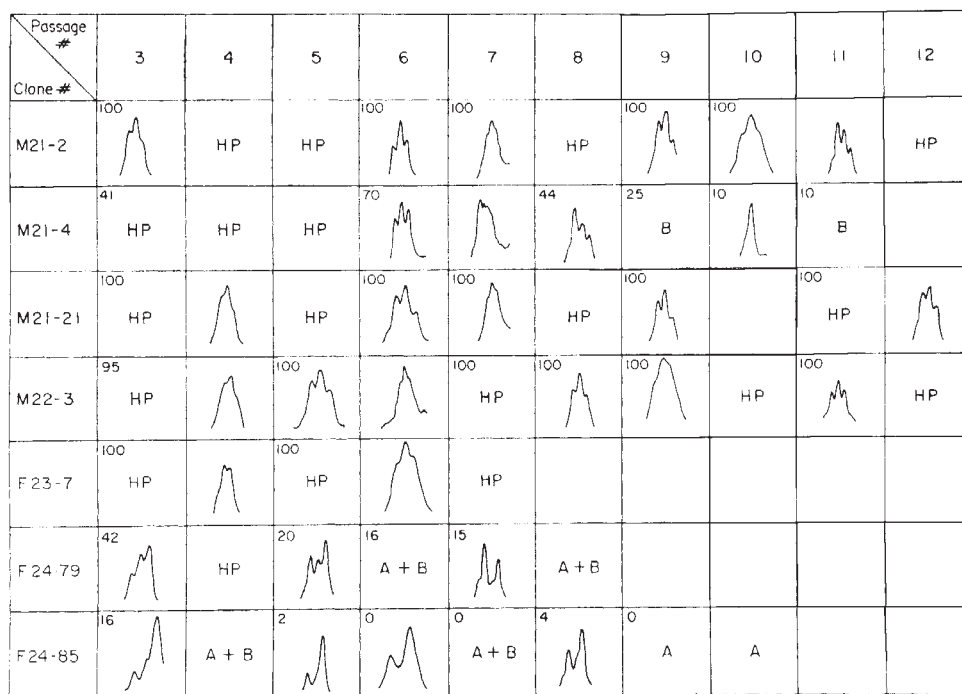


Fig. 2 Cytogenetics and electrophoretic profiles of individual hybrid isolates at various passage levels. Numbers express percentages of tetraploid metaphases per culture at a given passage. Densitometric tracings are from electrophoretic separations which were screened in a Beckman R-112 densitometer at 520 λ . Otherwise, the G6PD patterns were observed visually and recorded according to the judgment of three independent observers (HP, heteropolymer present; B, G6PD_B phenotype; A, G6PD_A phenotype; A + B, G6PD_A and G6PD_B phenotype, no heteropolymer).



no evidence that other mechanisms^{9,10} gave rise to mixoploidy in these colonies.

Of the total of nine isolates containing hybrids (Tables 1 and 2), one was lost due to contamination at the third *in vitro* passage; another consisted of non-replicating cells and, therefore, could not be carried beyond the initial diagnostic step. The seven remaining isolates were subjected to continuous *in vitro* passage and repeatedly analysed. As shown in Fig. 1, the initial growth rates of six of these cultures varied between 0.3 and 0.7 population doublings per day, which is within the usual range for phase II diploid fibroblast cultures in our laboratory. This growth pattern prevailed for an average of five passages, after which the cultures entered phase III of their *in vitro* lifespans (as shown by the irreversible drop of the estimated population doublings per day). Except for obviously random losses of one or two chromosomes and occasional broken and scattered metaphases, only the diploid or tetraploid modes of chromosome counts were observed in all of these cultures. As shown in Fig. 2, three initially 100% tetraploid isolates remained tetraploid throughout their entire *in vitro* lifespans, while also retaining the heteropolymer band.

Table 2 Summary of cytogenetic and electrophoretic classification of clonal isolates from 7 experiments involving a G6PD_{AB} heterozygote strain

Type of fusion:	Total clones screened/isolated (6,944/136*)			
	G6PD A	G6PD B	G6PD A + B	G6PD Heteropolymer
100% Diploid	6	4	1	
>90% Diploid	1	2	2	
Diploid + tetraploid	16	22	39	2
>90% Tetraploid	6	3	1	
100% Tetraploid	7	14		1

* 9 not tested.

Method as described for Table 1.

Deviations from the symmetrical 1:2:1 pattern seemed in most instances to reflect the evolution of subpopulations in mixoploid isolates. For example, the mixoploid isolates F24-79 and F24-85 (Fig. 2) lost the initial hybrid G6PD during serial culture, which was consistent with their respective cytogenetic evolutions.

These data provide no evidence that hybrids between diploid human fibroblasts regularly eliminate individual chromosomes or chromosome sets, as is the case with hybrids

derived from fusions, at least one partner of which is heteroploid. On the contrary, such hybrids seem to proliferate and undergo *in vitro* senescence as chromosomally stable, tetraploid cultures, provided the primary isolates were free of diploid contaminants.

A final point to be made is that the proliferative capacities of the hybrids between human diploid fibroblast strains is not different from other (non-hybrid) tetraploids which were 'aged' as controls, and compares favourably with the usual range of lifespans of moderately vigorous human fibroblast clones of diploid nature¹¹. Ultimately, however, the hybrid strains show the finite replicative potentials characteristic of their parents. There is, therefore, no evidence for a complementation of independent genetic defects which might be associated with some somatic mutational theories of clonal senescence¹². It would, of course, be of great interest to compare the longevities of a series of hybrid clones between parental strains of contrasting longevities, including those from patients with progeroid syndromes¹, although the poor growth of the progeroid parents would pose technical difficulties. The main application of the method, however, should come from the study of genetic complementation in the various human inborn errors of metabolism, which so far has been limited to heterokaryons¹³⁻¹⁵, in which there are uncertainties in interpretation. We believe that our non-selective approach can be used without difficulty for the isolation of hybrids between any given combination of euploid fibroblast strains and is able to provide, from a relatively modest group of isolates, quantities of karyotypically stable cells sufficient for a variety of biochemical studies.

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Carcinogenicity of the food additive furylfuramide in foetal and young mice

FURYLFURAMIDE (so-called AF-2), 2-(2-furyl)-3-(5-nitro-2-furyl) acrylic acid amide, had been used widely (probably several tonnes yr⁻¹) as an antimicrobial food additive in such food as fish sausage, mixed meat sausage, boiled fish paste and soybean curd throughout Japan until it was banned in 1974. Tonomura and Sasaki¹ found that furylfuramide induces chromosome aberrations in cultured human lymphocytes and strong mutagenicity was found by Kada² and Kondo and Ichikawa-Ryo³ with *E. coli*. Miyaji⁴ had reported, however, that neither tumour nor malformation was induced by furylfuramide in rats and mice, although Morris *et al.*⁵ reported that some other nitrofurans derivatives induced tumours in rats. Ames and his associates^{6,7} have shown that almost 90% of chemical carcinogens so far tested are mutagenic in their specified tester strains of *Salmonella typhimurium*. Thus, furylfuramide has been an important exception to the presumptive rule that a carcinogen is, in principle, a mutagen. As fish sausage has been favoured so much by young Japanese children, it was necessary to reopen the question of the carcinogenicity of furylfuramide in view of its strong mutagenicity. I report here that furylfuramide induces low but significant yields of tumours in mice when administered at the foetal or young stage.

The experiment with foetuses and newborns (Table 1) was conducted as follows. Virgin ICR/Jcl female mice (Japan Central Laboratory for Experimental Animals), 10 weeks old (27–30 g), were placed in a cage with a breeder male at 2200 h, and the next morning the vaginal plug was sought to determine day 1 of gestation. Sixteen pregnant mice (group 1) received three subcutaneous injections of furylfuramide (provided by Drs S. Odashima and I. Suzuki, National Institute of Hygienic Science, Tokyo) (dissolved in propylene glycol) at a dose of 50 µg per g body weight, once a day, on days 13, 15 and 17. Newborns of the 21 mother mice (group 2) received a subcutaneous injection of furylfuramide (50 µg per g body weight) within 12 h of birth, and three further injections were given on days 7, 14 and 21 after birth. As a solvent control (group 3), an equivalent

volume of propylene glycol was given to pregnant mice or newborns. These offspring were separated from mothers 4 weeks after birth, and maintained on mouse diet CA-1 (CLEA)⁸. They were killed at week 32 after birth, and pathological lesions were examined for tumours as described before^{9,10}.

Young mice (21 d old and weighing 9–11 g) were divided into five groups (Table 2). The first consisted of 57 male and 51 female ICR/Jcl mice which received three subcutaneous injections of furylfuramide (50 µg per g body weight), once a day, on days 21, 22 and 23 after birth. The second group (25 males and 35 females) received a single subcutaneous injection of furylfuramide at a dose of 100 µg per g body weight on day 21. Solvent controls (group 3) (61 males and 56 females) received an equivalent volume of propylene glycol on the same time schedule. As positive controls (groups 4 and 5), 7,12-dimethylbenz (a) anthracene (DMBA, provided by Drs Odashima and Suzuki) and urethane (Wako) were given to mice on day 21 after birth. Doses of these carcinogens correspond to the maximum tolerance doses in adult ICR/Jcl mice.

When furylfuramide was given to pregnant mice, a significant decrease of live births ($P < 0.001$, Table 1) was observed, and only one-third of them lived to the adult stage (8 weeks). The survivors, however, developed significantly higher incidence of lung tumours than controls, whereas tumours were not induced significantly in mice treated during the neonatal and suckling stages (Table 1). Histologically, most lung tumours were papillary adenomas. Three consecutive treatments of young mice with furylfuramide also induced a significantly high incidence of tumours, whereas a single injection did not (Table 2). It is not surprising, however, that Miyaji⁴ failed to detect the carcinogenicity of furylfuramide, because the incidence of tumours induced by furylfuramide was very low compared with the commonly used carcinogens DMBA and urethane (Table 2). Some tumours were induced in the forestomach but not in other organs of ddY/SLC mice after feeding on a diet containing furylfuramide for 18 months (Y. Ikeda, personal communication). This may be due to a high rate of decomposition of furylfuramide on the way to other internal organs after direct exposure on the stomach, and/or to the fact that adult mice are in general more resistant to various carcinogens than are foetal and young mice^{8,9}. When adult ICR/Jcl mice (10 weeks old and weighing 27–30 g) received three consecutive injections of furylfuramide (50 µg per g each time), lung tumours were not induced significantly (3 of 33 mice (9.1%); 0.12 ± 0.07 tumours per lung, not significant from controls ($P = 0.5$)). Furthermore, pretreatment of the young mice with phenobarbital (80 µg per g body weight), which is commonly used for induction of drug catabolising enzymes in the liver and other organs, suppressed the incidence of lung tumours in mice receiving three consecutive injections of furylfuramide (50 µg per g each time) from 29.1% to 4.8% (three of 63 mice treated with both phenobarbital and furylfuramide; 0.06 ± 0.04 tumours per lung, not significant from controls ($P = 0.8$) (T.N., unpublished)). The experiments reported here demon-

Table 1 Effects of furylfuramide on foetal and newborn mice

No.	Experimental groups		No. of pregnant mice	Live births No. (mean)	Incidence (%)	Lung tumours*			
	Age	Dose (µg g ⁻¹)				χ ² test†	Tumours per lung	t test‡	Others
1	Foetus	50 × 3	16	134 (8.4 ± 0.2)§	7/41 (17.1)	$P < 0.02$	0.22 ± 0.09 §	$P < 0.05$	3 OA
2	Newborn	50 × 4	21	234 (11.2 ± 0.2)	4/43 (9.3)	NS	0.09 ± 0.04	NS	2 OA, 2 TA
3	Control		7	81 (11.6 ± 0.5)	2/75 (2.7)		0.03 ± 0.02		

OA, Ovarian atrophy; TA, testicular atrophy; NS, not significant.

*Histologically papillary adenomas. Males and females were not separated, because there was no difference in the incidence of tumours.

†χ² test was applied with Yates' correction.

‡t test was applied with approximation of Cochran-Cox, if variance ratio was over *F* value at 1%.

§Mean ± s.e.

Table 2 Carcinogenicity of furylfuramide on young mice

No.	Experimental groups	Dose ($\mu\text{g g}^{-1}$)	No. of mice at start	Tumour-bearing mice* Incidence (%)	χ^2 test†	Incidence (%)	Lung tumours* χ^2 test†	Tumours/lung	<i>t</i> test‡	Others
1	Furylfuramide	50 × 3	108	17/55 (30.9)	$P < 0.001$	16/55 (29.1)	$P < 0.001$	0.33 ± 0.07§	$P < 0.001$	2 TA, 1 OC
2	Furylfuramide	100	60	1/34 (2.9)	NS	1/34 (2.9)	NS	0.03 ± 0.03	NS	
3	Control		117	2/92 (2.2)		2/92 (2.2)		0.02 ± 0.02		
4	DMBA	20	80	57/62 (91.9)	$P \ll 0.001$	50/62 (80.6)	$P \ll 0.001$	14.3 ± 2.6	$P \ll 0.001$	15 L, 7 F, 2 P 2 SH, 21 OA
5	Urethane	1500	40	32/35 (91.4)	$P \ll 0.001$	30/35 (85.7)	$P \ll 0.001$	16.7 ± 2.5	$P \ll 0.001$	1 L, 2 LH, 1 OC

TA, Testicular atrophy; OA, ovarian atrophy; OC, ovarian cystadenoma; L, thymic lymphoma; F, fibrosarcoma (injection site); P, papilloma of skin; SH, splenic haemangioma; LH, liver haemangioma; NS, not significant.

*Mice were killed at week 27 after treatment. Males and females were not separated, because there was no difference in the incidence of tumours.

† χ^2 test was applied with Yates' correction.

‡*t* test was applied with approximation of Cochran-Cox, if variance ratio was more than the *F* value at 1%.

§Mean ± s.e.

strate that even though a chemical has been reported to be non-carcinogenic, if it is later found to be mutagenic there is good reason to re-examine its carcinogenicity in a more sensitive test, such as treatment of foetal or young mice. Mutagenicity in microorganisms is certainly a good indicator for selecting environmental chemicals to be submitted to long term carcinogenicity testing.

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Chromosome aberrations induced in rat lymphocytes by N-nitroso compounds as a possible basis for carcinogen screening

MANY N-nitroso compounds are toxic, carcinogenic, mutagenic and teratogenic, and several are known to give rise to alkylating intermediates in the body¹. The nitrosamines (for example, dimethylnitrosamine) require activation by enzymes before yielding these intermediates but nitrosamides (for example, N-methylnitrosourea) may react with nucleic acids and proteins without metabolic activation. The nature of the various intermediates between the nitroso compound and the intracellular methylating agent has not been established, however, and the question of the duration of their existence in the body arises. With dimethylnitrosamine, the acute lesion is mainly severe necrosis of the centrilobular zones of the liver, including damage to the hepatic sinusoids and central veins as well as to the centrilobular parenchymal cells^{2,3}. It therefore follows that at least one of the intermediates must be of sufficient stability to damage the vascular cells. Since dimethylnitrosamine is active in the host-mediated assay for mutagenesis⁴ an active intermediate must also be capable of passing into the

peritoneal cavity. N-methylnitrosourea is mutagenic in bacteria⁵ and is known to induce chromosomal aberrations in mammalian and plant cells *in vitro*^{6,7}.

This report describes the induction of aberrations in the lymphocytes of rats treated with dimethylnitrosamine, presumably during their passage through the liver and other organs capable of activating the compound.

As expected, there was a highly significant increase in the number of abnormal cells, that is, those cells containing either chromosomal aberrations or achromatic gaps, from lymphocyte cultures treated *in vitro* with N-methylnitrosourea (0.9 mM), as compared with the abnormal cells from the untreated controls ($\chi^2 = 26.6$, 1 d.f., $P < 0.0005$, abnormal cells containing achromatic gaps have been included in the calculation of significance levels given in the text. Significance levels based on data excluding achromatic gaps are given in Table 1). N-methylnitrosourea seems to induce chromatid type breaks, the vast majority of our aberrations being similar to those already reported for this compound in experiments with *Vicia*⁷, that is, chromatid breaks, chromatid exchanges and isochromatid breaks. We did find, however, two possible dicentric, neither of which was accompanied by fragments, in the 200 treated cells. There were no dicentric in the controls.

This experiment served as a positive control for our next experiment in which rat lymphocyte cultures were treated with dimethylnitrosamine (0.9 mM) *in vitro*. There was no significant difference between the cultures treated *in vitro* with dimethylnitrosamine and the control cultures ($\chi^2 = 1.23$, 1 d.f., $P > 0.2$). In our next experiments we cultured lymphocytes from two animals (A and B) who had been given dimethylnitrosamine 30 mg per kg body weight by intraperitoneal injection, 6 h before being killed. The total numbers of abnormal cells from these animals were in each case significantly higher than the level of their respective controls (animal A: $\chi^2 = 10.75$, 1 d.f., $P < 0.005$; animal B: $\chi^2 = 7.87$, 1 d.f., $P = 0.005$). In these experiments with animals A and B the breaks were again principally of the chromatid type. The significance of these chromatid type aberrations will be discussed later.

Our results clearly indicate that the active intermediate(s) formed metabolically from dimethylnitrosamine have sufficient stability to pass from their sites of formation in the liver and other organs capable of activating the compound and probably to reach the nuclei of the circulating lymphocytes. Since the majority of known chemical carcinogens require metabolic activation^{8,9} similar effects on lymphocytes might be expected in animals exposed to them. We are carrying out experiments with other types of carcinogen to investigate this possibility. If this procedure could be developed into a method for carcinogen screening it would have some advantages because the test object is a mammalian cell which is exposed to the metabolic systems for activation of carcinogens by the intact animal rather than the various cellular and subcellular *in vitro* pre-

Table 1 Numbers of rat lymphocytes with chromosome aberrations or achromatic gaps found after *in vitro* treatment with N-methylnitrosourea or dimethylnitrosamine, or *in vivo* treatment with dimethylnitrosamine

	No. of cells scored	Metaphase cells with chromosome aberrations	Metaphase cells with achromatic gaps	Cells scored by two observers (L.L. or B.B.)	Significance of difference between treated and untreated cultures (excluding cells with achromatic gaps)
N-methylnitrosourea (0.9 mM) <i>in vitro</i>	100	18	3	LL	} $\chi^2 = 19.56$ 1 d.f., $P < 0.0005$
	100	22	2	BB	
Control	100	3	5	LL	
	100	2	0	BB	
Dimethylnitrosamine (0.09 mM) <i>in vitro</i>	100	1	0	LL	} $\chi^2 = 0.42$ 1 d.f., $P < 0.60$
	100	3	1	BB	
Control	100	2	3	LL	
	100	4	1	BB	
Dimethylnitrosamine (30 mg per kg body weight, <i>in vivo</i> (animal A))	100	10	2	BB	} $\chi^2 = 6.28$ 1 d.f., $P < 0.025$
	100	11	5	LL	
Control	100	4	1	BB	
	100	4	1	LL	
Dimethylnitrosamine (30 mg per kg body weight, <i>in vivo</i> (animal B))	100	9	2	LL	} $\chi^2 = 9.60$ 1 d.f., $P < 0.005$
	100	11	1	BB	
Control	100	3	1	LL	
	100	2	2	BB	

Blood was collected from the abdominal aorta of female Wistar rats, cultured and collected as described¹² previously but with the substitution of foetal bovine serum for human AB serum and the addition of glutamine. In the *in vivo* experiments rats were injected intraperitoneally with dimethylnitrosamine, 30 mg per kg body weight, 6 h before the collection of blood. In the *in vitro* experiments blood was cultured for 24 h before the addition of dimethylnitrosamine, dissolved in 0.9% NaCl, or N-methylnitrosourea, dissolved in 0.01 M citrate buffer, pH 6.7, to give final concentrations of 0.9 mM. In both the *in vitro* and the *in vivo* experiments, the cultures were collected at 48 h and slides were coded for blind scoring.

parations currently in use. The sensitivity of the method might be improved by using a sister chromatid exchange method of scoring¹⁰.

We have mentioned that chromatid type breaks were formed after the treatment of rats *in vivo*. Such treatments, of lymphocytes in the G₀ stage of division before DNA synthesis, might be expected to result in chromosome type aberrations appearing in culture, after stimulation. There is evidence, however,—for example, the work of Yoder *et al.*¹¹ on agricultural workers exposed to pesticides—that exposure *in vivo* can indeed lead to the production of chromatid type aberrations at the first division in culture. It would be interesting to know if the active agent can induce lesions in the G₀ chromosomes which will later be manifested as chromatid-type aberrations, or whether the active agent survives long enough to be carried over, with or in the lymphocytes, to act during the DNA synthesis which follows stimulation in culture. If, however, the active agent should prove to be the methylcarbonium ion then it is difficult to see how the latter hypothesis could be correct.

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Altered chromatin ultrastructure in neutrophils of schizophrenics

IN spite of reports of altered nuclear morphology of blood cells^{1–3} and evidence advocating the participation of genetic factors in schizophrenia^{4,5}, little attention has been paid to the possibility that altered chromatin is associated with this mental illness. Since research in this area may further the understanding of the mechanisms underlying the disease, we compared the chromatin of schizophrenics and normal subjects^{6,7}, using the neutrophil granulocyte as a model of fully differentiated, non-dividing cells with fixed heterochromatinisation. Our approach was based on current knowledge of chromatin^{8–10} and focused on the particular roles of the various histone fractions in determining chromatin structure and function^{11,12}. The results obtained from the initial study⁸ showed that schizophrenics differ from controls in their nucleohistone staining pattern. This is characterised mainly by an increased availability of arginine-rich histones⁷ to the anionic phosphotungstic acid-haematoxylin (PTAH) staining reagent¹³. We interpreted this as indicating an increased readiness of the nucleohistones in the schizophrenics to undergo conformational changes leading to unmasking of such proteins. Considering the role of lysine-rich histone H1 in cross linking the chromatin^{12,14}, we tentatively proposed that in schizophrenics histone H1 tends to dissociate more readily from the chromatin complex.

To relate these findings to chromatin fine structure we adapted a histochemical procedure, previously used for

smears⁷, to electron microscopy (M.R.I. and T.K., manuscript in preparation). We took advantage of the presence of the heavy metal-phosphotungstic acid (PTA) complex, a protein stain for basic residues¹³, in the PTAH anionic stain. This could serve as the 'electron stain' and duplicate the histochemical reaction at the ultrastructural level.

We used neutrophil nuclei from 10 schizophrenics and 10 normal controls. All patients were drawn from an experimental group of chronic inpatients at the Corfu State Psychiatric Hospital, who had been drug-free for more than a year and well stabilised in their mental state. They were physically healthy without haematological abnormalities. Controls were physically and mentally healthy volunteers selected from the hospital's resident personnel on the basis that they met the matching criteria on sex and age and shared with the patients more or less the same living conditions and diet. Peripheral blood was obtained on the same day and in the same conditions from patients and controls. EDTA (Versene, 1 drop per 2 ml blood) was used as the anticoagulant. Samples were coded and processed 'blind'. The erythrocytes were permitted to sediment by

Fig. 1 Electron micrographs of human neutrophil granulocytes. *a*, Control subject, condensed chromatin in cell appears white ($\times 7,300$). *b*, Schizophrenic subject, condensed chromatin in cell appears black ($\times 7,300$). Both sections are from pellets of cells prepared identically by fixing for 30 min in 2.5% cold glutaraldehyde (buffered with 0.2 M cacodylate), rinsing in cold buffer (twice, 1 h each), treating with 0.25% aqueous KMnO_4 at 4 °C for 5 min, rinsing in distilled water, bleaching with 5% oxalic acid for 10 min and washing in tapwater. The pellets were stained in bulk with PTAH (1 g haematoxylin, 20 g PTA 1,000 ml distilled water, 0.177 g KMnO_4) for 48 h at room temperature. Dehydration was carried out in a graded series of ethyl alcohol; after two changes of propylene oxide the pellets were embedded in Epon 812. Thin sections were viewed, without further staining, in a Phillips EM 200 electron microscope. In (*a*) two of the lobes are joined by a "nuclear filament" (double-headed arrow) seen as a nuclear envelope-limited sheet. Its strong uptake of PTA indicates a high availability of basic groups in the proteins. The arrow indicates the wavy layer of extended chromatin.

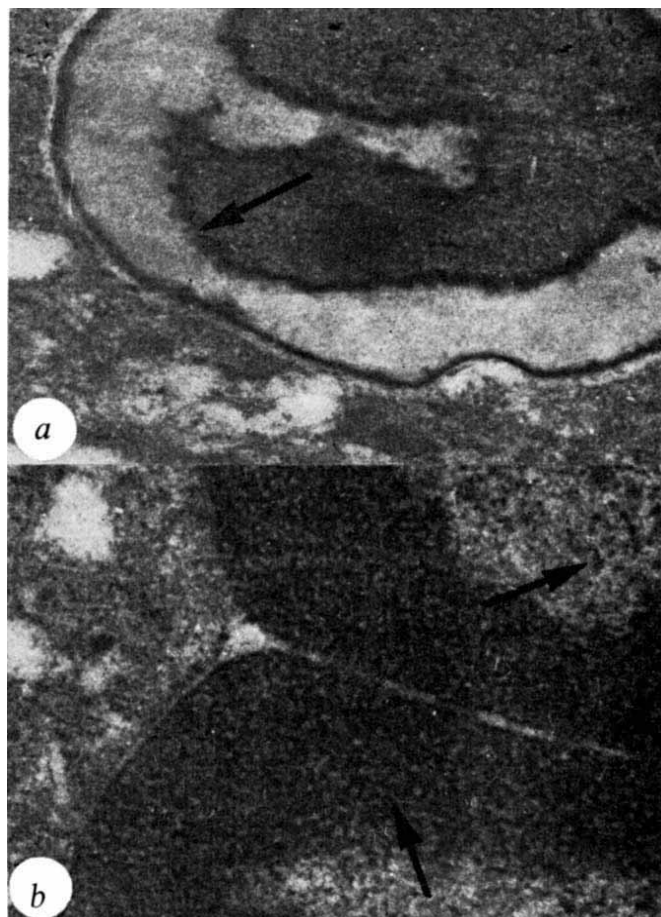
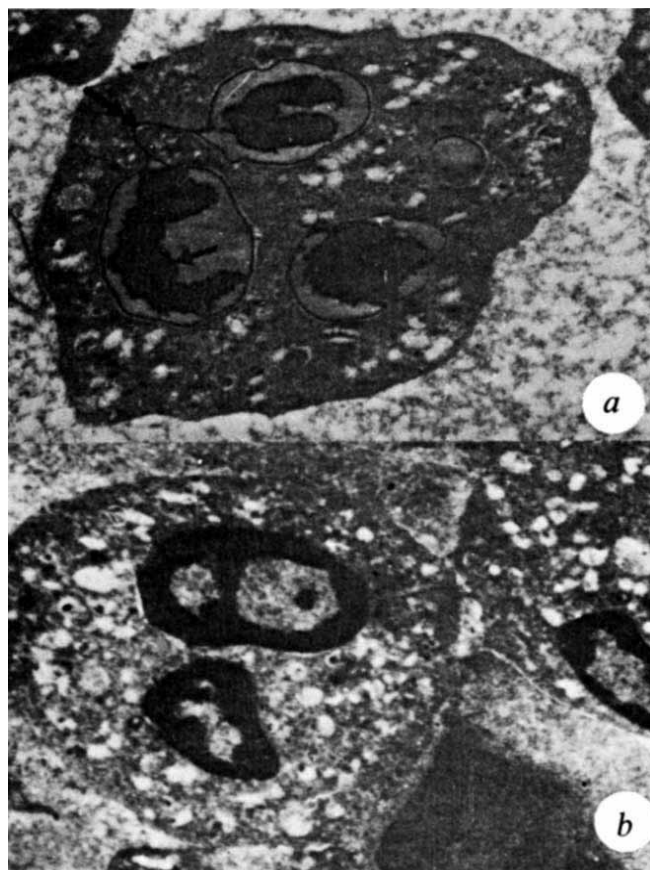


Fig. 2 Electron micrographs of human neutrophil granulocytes. *a*, Control subject, compact chromatin appears white ($\times 42,900$). *b*, Schizophrenic subject, compact chromatin appears black ($\times 42,900$). The cells were prepared as in Fig. 1. The fine structure of the cytoplasm in both (*a*) and (*b*) is identical. In (*a*) the arrow indicates an extended chromatin layer and in (*b*) the arrows indicate individual chromatin fibres.

gravity for 1 h. The leukocyte-rich plasma was removed and kept refrigerated for 24 h. The cells were pelleted and prepared for electron microscopy by glutaraldehyde fixation (see legends to Figs 1 and 2). Double fixation for blood cells with glutaraldehyde and osmium¹⁶, followed by uranyl acetate postfixation¹⁷, was also used. The Epon sections were stained further by uranyl acetate and lead citrate¹⁷.

The PTAH-in-bulk preparatory procedure, but not the osmium-uranium-lead method, revealed differences between patients (Fig. 1*b*) and controls (Fig. 1*a*). In all normal subjects, with the exception of one (Table 1), the area of condensed chromatin of all neutrophils had a clear ground-glass appearance, signifying non-binding of the anionic stain. The dispersed template-active chromatin, however, extending around the periphery of the compact areas (Figs 1*a* and 2*a*, arrows) appeared black, signifying strong PTA binding. Since the basic groups (lysine and arginine residues) are believed to be the binding sites for PTA, these

Table 1 Distribution of subjects according to the pattern of condensed chromatin ultrastructure in their neutrophils

Groups	Type a*	Type b†	Total	χ^2	<i>P</i>
Controls (<i>n</i> = 10)	9	1	10	16.36	0.001
Schizophrenics (<i>n</i> = 10)	0	10	10		

*Condensed chromatin which appears white in Figs 1 and 2.

†Condensed chromatin which appears black in Figs 1 and 2.

results can be understood to depend on the steric configuration of the histone molecules in the nucleoproteins^{15,18} and the interaction of the chemicals used (EDTA, KMnO₄) with the chromatin^{11,19}. A narrow zone around the circumference of the nucleus also showed strong PTA binding and is continuous with the nuclear filament connecting the two lobes of the neutrophil¹⁶ (Fig. 1a, double-headed arrow). In contrast to controls, the neutrophils of schizophrenics all had, in the area of condensed chromatin (Table 1), a uniformly high PTA density signifying strong binding of the anionic stain (Fig. 1b). The fine structure of the chromatin was sharply outlined as a skein of tortuous fibres 100–300 Å in diameter (Fig. 2b arrows). The ultrastructure of the nuclear sap showed a finely granular fairly homogeneous density in the controls, whereas in the schizophrenics it was coarsely fibrous with a light background. Although the preparatory procedure and techniques used were the same for the samples of both groups, the area of compact chromatin in the micrographs appears unstructured white in the controls (Figs 1a and 2a) and fibrous black in the schizophrenics (Figs 1b and 2b). This staining pattern indicated that steric hindrance is responsible for the lack of PTA uptake in the nucleoprotein of the controls, probably determined by the state of compaction of the chromatin¹⁸. Thus the high uptake of PTA in the schizophrenics denotes less compact heterochromatin. This altered state of the chromatin is similar to that observed in the early stages of nuclear maturation¹⁸ when chemical modifications in histones, such as phosphorylation, facilitate their dissociation from the chromatin²⁰. Although preparative procedures can cause light and dark appearances of the chromatin in the same cell type¹⁸, the methodological precautions taken in our study rule out this possibility. The differences observed can only be attributed to biological factors.

We conclude that in the neutrophil nuclei of schizophrenics there is a tendency for the chromatin to respond to decondensing stimuli by undergoing conformational changes. If histone H1 can be viewed as the condensing factor of the chromatin^{11,12,14} then weak binding due to increased phosphorylation²⁰ may be considered to be a possible molecular basis of this susceptibility to stimuli leading to the altered state we observed in chromatin. Should further work establish the validity of this postulate, various biochemical findings in schizophrenia (for review see ref. 21) could be explained on the basis of an altered genomic expression and might be unified by a common denominator residing in the chromatin at the interface of gene and environment.

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Different Ly antigen phenotypes of *in vitro* induced helper and suppressor cells

THYMUS-derived (T) cells have a major role in immune systems. They mediate various functions, such as T-B cooperation ('helper cells') and the mixed lymphocyte reaction², become killer cells³, are active in graft *versus* host responses (reviewed in ref. 4) and act as suppressor cells⁵. The identification of the cells responsible for these functions as T cells was facilitated by the use of antisera against the T-cell-specific alloantigen, Thy-1 (ref. 6) (formerly known as θ). It has been reported that Ly alloantigens, present exclusively on T cells⁷, identify functionally distinct subpopulations of T cells⁸. For example, T helper cells were lysed by anti-Ly-1, but not by

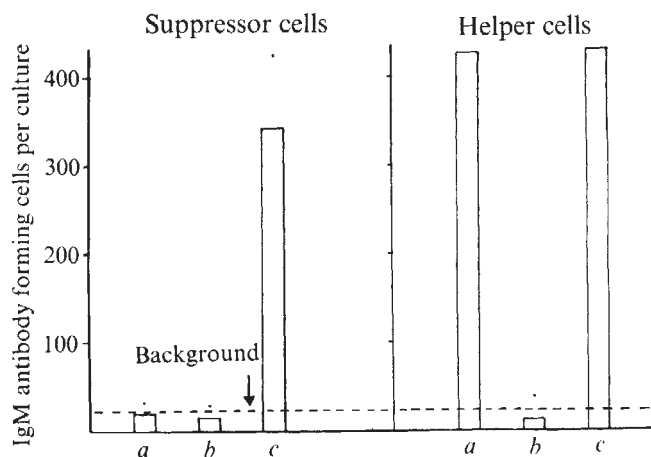


Fig. 1 Effect of anti-Ly sera and complement on specific helper and suppressor cells generated *in vitro* from CBA spleen cells. *a*, Complement; *b*, complement and anti-Ly-1.1; *c*, complement and anti-Ly-2.1. Helper cells were induced as described in ref. 12. Spleen cells were cultured in Marbrook flasks with keyhole limpet haemocyanin (KLH), 0.1 µg ml⁻¹ for 4 d with Eagle's MEM containing 5% foetal calf serum. The surviving cells, termed 'helper cells' were assayed for their capacity to cooperate with TNP reactive B cells present in normal CBA spleen in the presence of TNP KLH. Anti-DNP or TNP (di- or trinitrophenyl) antibody-forming cells were assayed using hapten-conjugated anti-SRC Fab' fragments coupled to sheep red cells (SRC) using the Cunningham plaque assay¹⁴. Suppressor cells were induced as in ref. 14, culturing (as above) with KLH (100 µg ml⁻¹) for 4 d. Suppressor cells (T cells as judged by the effect of anti-Thy-1 or heterologous anti-T) were assayed by their capacity to inhibit the helper effect of 3 × 10⁶ helper cells. Specificity tests of the suppressor effect are described in ref. 14. Suppressor or helper cells were treated with anti-Ly-1.1 at 1/50 dilution or anti-Ly-2.1 at 1/40, washed and treated with absorbed rabbit complement at 1/5. After treatment cells were counted in Trypan blue. In helper experiments 10⁵–3 × 10⁶ viable cells were assayed while in suppressor experiments 10⁴–10⁶ viable cells were added to 3 × 10⁶ helper cells for assay. For simplicity, results of one cell dose only (3 × 10⁶ helpers, 10⁵ suppressors) are shown. Four experiments of this type have been performed. Anti-Ly-2.2 at 1/15 has also been found to inhibit suppressor cells from C57BL (Ly-1.2, 2.2) mice (two experiments, data not shown). Background antigen (TNP KLH) in the absence of helper cells. Dots above bars represents value and 1 s.e.

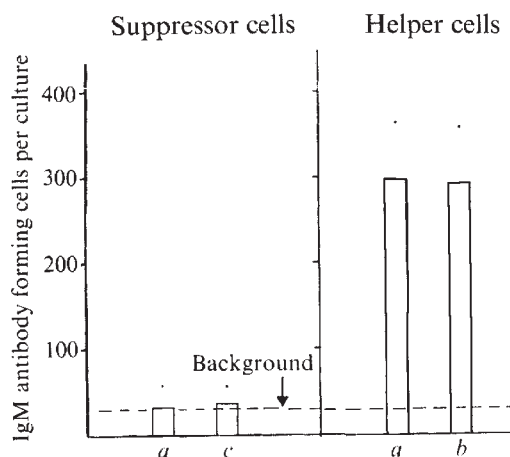


Fig. 2 Functional tests for the specificity of the anti-Ly-1.1 and anti-Ly-2.1 antisera used T helper and suppressor cells raised as described (Fig. 1) from C57BL mice, which like the CBA/Ca were obtained from the ICRF animal unit at Mill Hill, London. Cells were treated with anti-Ly-1.1 or 2.1 and complement as described in the legend to Fig. 1, and 3×10^5 viable helper cells with or without 10^5 suppressor cells were placed in the second culture. No inhibition occurred, verifying functionally the serological specificity of the antisera. *a*, *b* and *c* have the same meaning as in Fig. 1.

anti-Ly-2, so that their phenotype is $Ly-1^{+2^{-}}$, whereas T killer cells were a distinct subpopulation, being $Ly-1^{+2^{+}}$. We report here that specific T suppressor cells induced *in vitro* have a different Ly alloantigen phenotype from T helper cells induced *in vitro* from the same spleen cell pool.

Anti-Ly-1.1, anti-Ly-2.1 and anti-Ly-2.2 antisera were prepared as described before⁹. They were tested for titre and specificity in a two-stage cytotoxic test using rabbit complement, absorbed with mouse spleen cells. If necessary, sera were absorbed with thymocytes of appropriate

Ly phenotype to remove autoantibody. Sera were used at dilutions giving plateau levels of cytotoxicity.

The effect of anti-Ly-1.1, anti-Ly-2.1 and complement, or complement alone on *in vitro* induced helper cells of the CBA (Ly phenotype $Ly-1^{+}$, 2.1^{+}) mouse strain is shown in Fig. 1. Anti-Ly-1.1 abolished helper activity. Anti-Ly-2.1 had no suppressive effect, confirming the results of Kieselow *et al.*⁸. The effect of the same antisera on specific suppressor T cells, derived from the same pool of spleen cells as the helper cells, is also shown in Fig. 1. Suppressor cells were lysed by anti-Ly-2.1 but not by anti-Ly-1.1. Since the above experiment was not performed with Ly congenic mice, we attempted to verify that the observed differential effect of anti-Ly-1 and anti-Ly-2 antisera were due to their content of anti-Ly-1.1 and anti-Ly-2.1 antibody. First, the anti-Ly-1.1 and anti-Ly-2.1 antisera were tested on populations of helpers and suppressor cells derived from the C57BL strain which bears the opposite Ly alleles— $Ly-1.2$ and $Ly-2.2$. They had no effect (Fig. 2). Further evidence of the specificity of the antisera came from absorption studies. Anti-Ly-1.1 antiserum was absorbed with mixed spleen and thymus cells from C57BL/10 ($Ly-1.2$, 2.2) or C57BL/6 $Ly-1.1$, and anti-Ly-2.1 was absorbed with C57BL/10 or C57BL 75NS ($Ly-1.2$, 2.1). The absorbed antisera were tested for their effects on CBA ($Ly-1.1$, 2.1) helper and suppressor cells. Figure 3 shows that the relevant antibody activity was absorbed as expected, verifying that the antibody activity lysing the suppressor cells was anti-Ly-2.1.

These results demonstrate unequivocally that specific T suppressor cells differ from specific T helper cells, even if both are induced from the same spleen cell pool, in similar *in vitro* conditions, and directed to the same antigen, keyhole limpet haemocyanin. This has three implications. First, it demonstrates that the mechanism of specific T-cell suppression, in this (but not in the other) model, is not due to excess help¹⁰. Second, it provides a means of selectively manipulating T-cell effects in antibody production (treatment with anti-Ly-2 antiserum has been shown to augment helper activity *in vitro*¹¹). Third, our data increase the evidence that differentiated T effector cells have distinctive Ly phenotypes. The relationship of the specific suppressor cells described here to cytotoxic T cells, with the same Ly phenotype, and to nonspecific suppressor cells characterised as $Ly-1^{+2^{+}}$ (ref. 11) remains to be clarified.

We thank Dr Ian McKenzie for breeding pairs of C57BL/6 $Ly-1.1$ and of C57BL 75NS mice, and Dr M. B. Rittenberg for keyhole limpet haemocyanin. We thank Dr Ullrich Hammerling, Memorial Sloan-Kettering Cancer Center, New York, for anti-Ly antisera.

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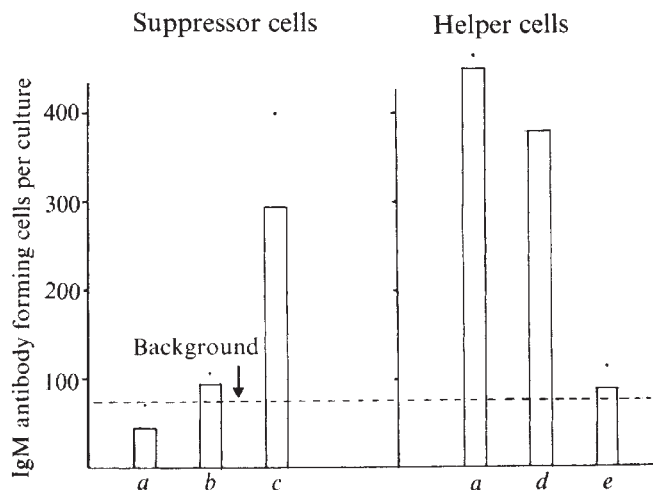
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Fig. 3 Absorption test of anti-Ly-1.1 and anti-Ly-2.1 with C57BL/6 $Ly-1.1$ and C57BL/675NS ($Ly-1.2$, 2.1 , 3.1). These mice are congenic to C57BL/6, were obtained from Dr Ian McKenzie, Auston Hospital, Melbourne, Australia and were initially derived by Dr M. Cherry. In this experiment suppressor and helper cells were treated with absorbed antisera (see legend to Fig. 1) and 3×10^5 viable helper cells with or without 10^5 suppressor cells placed in the second culture. *a*, complement; *b*, complement and $Ly-2.1$ absorbed with B6 $Ly-1.1$; *c*, complement and anti-Ly-2.1 absorbed with B10; *d*, complement and anti-Ly-1.1 absorbed with B6 $Ly-1.1$; *e*, complement and $Ly-1.1$ absorbed with B10.



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Surface charge modifications associated with proliferation and differentiation in neuroblastoma cultures

THERE have been several reports that malignant cells carry on their surface a higher density of negative fixed charges than normal cells^{1–3}. But it has been argued that the differences in observed surface charge reflect differences in cell growth rate rather than malignancy *per se*^{4–6}. It has been difficult to resolve this controversy because earlier studies involved comparison between different types of cells, or between cells from the same origin but grown in different culture conditions and at different times after seeding. Using a neuroblastoma cell line⁷, we have avoided these shortcomings and made electrophoretic measurements on cells which are homologous from the genetic viewpoint, and which are grown in the same culture tray and collected at the same time, but which exhibit different stages of morphological differentiation.

When neuroblastoma cells are seeded into a Petri dish containing normal culture medium (that is, medium with 10% calf serum), they divide rapidly during the logarithmic phase. Eventually they reach confluence, and contact inhibition causes the culture to enter a stationary phase. Neuroblastoma cells in the stationary phase differentiate and extend processes, and the small, round cells which characterise neuroblastoma tumours *in vivo*⁸, as well as the cloned cell line⁹ we used, are replaced by large cells, with longer processes, which are more differentiated and resemble sympathetic nerve cells^{7–10}. The stationary culture level, however, is not reached in synchronised fashion simultaneously over the entire tray. The cells typically appear in colonies^{7,8,10}, each with central differentiated cells, with the smaller, round and undifferentiated cells often occupying the periphery (Fig. 1a). Thus, in neuroblastoma cultures it is possible to explore differentiated, neurone-like cells, with extensive neurites (Fig. 1b), as well as undifferentiated, small, round cells without processes, which are actively dividing (Fig. 1c). Both cell types originate from the same seeded cells and thus are genetically homologous; they also bathe in the same culture medium and are collected at the same time, assuring uniformity of the environment. In the experiments described here, cells were collected 2–4 d after the initial seeding. Cells were separated from the Petri dish by directing a fine stream of medium. Cells were then suspended in the medium in which they were cultured previously, and introduced into a cylindrical microscopic electrophoresis apparatus^{11,12} (Rank Bros). Measurements were made at 37 °C, and at electric field strength of 2 V cm⁻¹. Care was taken to ensure that all measurements were performed in the stationary layer of the electrophoresis chamber¹². The pH was not controlled during electrophoresis, but the pH indicator incorporated in the medium indicated that no gross pH shift took place.

Although phase contrast optics have not been used, discrimination between differentiated and undifferentiated cells while the measurements were being taken in the electrophoresis chamber presented no difficulty since undifferentiated, dividing cells as a rule are less than 10 µm in diameter (Fig. 1c), whereas differentiated cells generally

vary from 20 to 30 µm in diameter, excluding their processes (Fig. 1b). To prevent possible error, we restricted measurements to two groups of cells: 8–10 µm in diameter, and more than 25 µm in diameter.

The results are summarised in the first column of Table 1. Clearly the large differentiated cells with processes migrate in an electric field with considerably lower velocity than the small, undifferentiated dividing cells. Both cell types are attracted to the positive electrode, indicating negative zeta potential. Zeta potentials calculated from electrophoretic mobility measurements represent only a lower bound on the total surface charge^{13,14}, the remainder of the charge being neutralised by adsorption of divalent and polyvalent cations; however, our experiments involve cells grown in the same culture medium in identical conditions, so that adsorption would be likely to be uniform

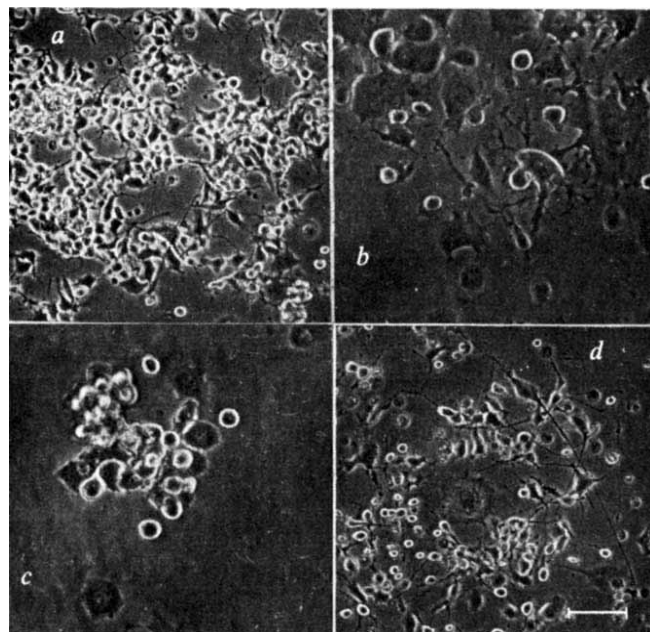


Fig. 1 Neuroblastoma cells in tissue culture. The N-18 clone of C-1300 mouse neuroblastoma cell line was seeded into plastic Petri dishes of 100 mm diameter (Falcon), and grown in Dulbecco's medium containing sodium pyruvate (110 mg l⁻¹). Cultures were incubated throughout at 37 °C, in equilibrium with 5% CO₂ in air. Seeding was adjusted so that at collection the cells would form colonies, but would not reach confluence. Phase contrast photographs of unfixed material. Scale: 100 µm for (a and d), and 50 µm for (b and c). Photographs (a–c) were taken from a culture 2 d after initial seeding, with 10% calf serum in the medium. Photograph (d) was taken from a culture 4 d after seeding, grown for the last 2 d with no serum in the medium.

for both differentiated and undifferentiated cells. Moreover, for the study reported here it is not necessary to calculate the actual zeta potential, since the measurements on both types of cell were made in identical ionic environment and temperature, and since the electrophoretic mobility measured is likely to be directly proportional to the total surface charge¹³. It is possible to conclude directly from Table 1 that the surface charge of undifferentiated cells is approximately 30% more negative than that of differentiated cells from the same culture. A *t*-test on our data indicates that the probability of this occurring by chance is below 0.5%. But, the possibility of systematic error remains. We have found the larger cells to be slower, but for particles with diameters more than 100 times the thickness of the ionic double layer (as have all living cells), the viscosity of the medium has no effect¹⁵. A more serious

difficulty is that while both cell types were collected from the same culture at the same time, the large, differentiated cells have probably been in existence considerably longer than the small, undifferentiated cells which are constantly dividing^{7,8}, making it necessary to distinguish the effect on the surface charge of cell proliferation from that of the degree of differentiation. This was possible in experiments with cells grown in medium devoid of serum.

Table 1 Electrophoretic mobility of neuroblastoma cells in normal medium and in serum-free medium

Diameter of cell	Normal medium	Serum-free medium
8–10 μm (undifferentiated)	1.27 (± 0.05)	1.26 (± 0.03)
25–40 μm (differentiated)	0.96 (± 0.04)	1.29 (± 0.06)

Mobility is expressed in $\mu\text{m s}^{-1}\text{cm V}^{-1}$, and the standard error is given in parentheses next to each measurement.

As reported before, neuroblastoma cells grown in medium not containing serum stop dividing and extend processes^{9,10,16}. This was true also in our cultures, and a large percentage of the cells are differentiated, with the cell body more than 25 μm diameter, and with numerous long neurites; nonetheless, there remained some small (<10 μm) round cells in these cultures (Fig. 1d). Thus it was possible to compare differentiated and undifferentiated cells also in cultures in which cell division was not occurring. The results are given in the second column of Table 1. Clearly the velocity of electrophoretic migration of differentiated and undifferentiated cells in serum-deprived medium is nearly identical.

It might be argued, however, that the differences in surface charge found in serum-containing media reflect differences in adsorption of serum on the surface of dividing and differentiated cells. According to this view, underlying structural differences in membrane structure may persist after withdrawal of the serum, but cannot then be detected electrophoretically.

To examine this possibility, we arrested cell division in the presence of a normal concentration of serum. Cells were grown initially in normal medium containing 10% foetal calf serum. Two days after seeding, cytosine arabinoside ($2 \mu\text{mol l}^{-1}$) was added to the medium without changing the concentration of the serum. The cells were collected after two further days of incubation in this medium (4 d after seeding). Cultures treated in this way contained both differentiated and undifferentiated cells, but the overall cell density was lower than in cultures grown in serum-containing medium. Cell electrophoresis was performed on cells treated in this way, as well as on control cultures seeded at the same time, but not treated with cytosine arabinoside. No significant difference was found between treated and untreated differentiated cells. The migration velocity of small, undifferentiated cells, however, is reduced by approximately 20% compared with their untreated counterparts. Since cytosine arabinoside is known to arrest cell division, these results support the initial conclusion from the experiments without serum, that the surface charge of the cell membrane is strongly negative shortly after cell division and becomes gradually more positive after the last cell division.

The results reported here suggest that cell proliferation, rather than the level of differentiation, is the significant factor determining surface charge of living cells. Specifically, our data indicate that a high rate of cell division is associated with increased negativity of the cell surface. Considered together with observations on the resting potential of dividing and non-dividing cells (unpublished results of R. E. and Y. Pri-Paz) the results reported here suggest a significant difference in cell membrane structure and function between proliferation and non-

proliferating cells from the same cell line, regardless of the level of morphological differentiation.

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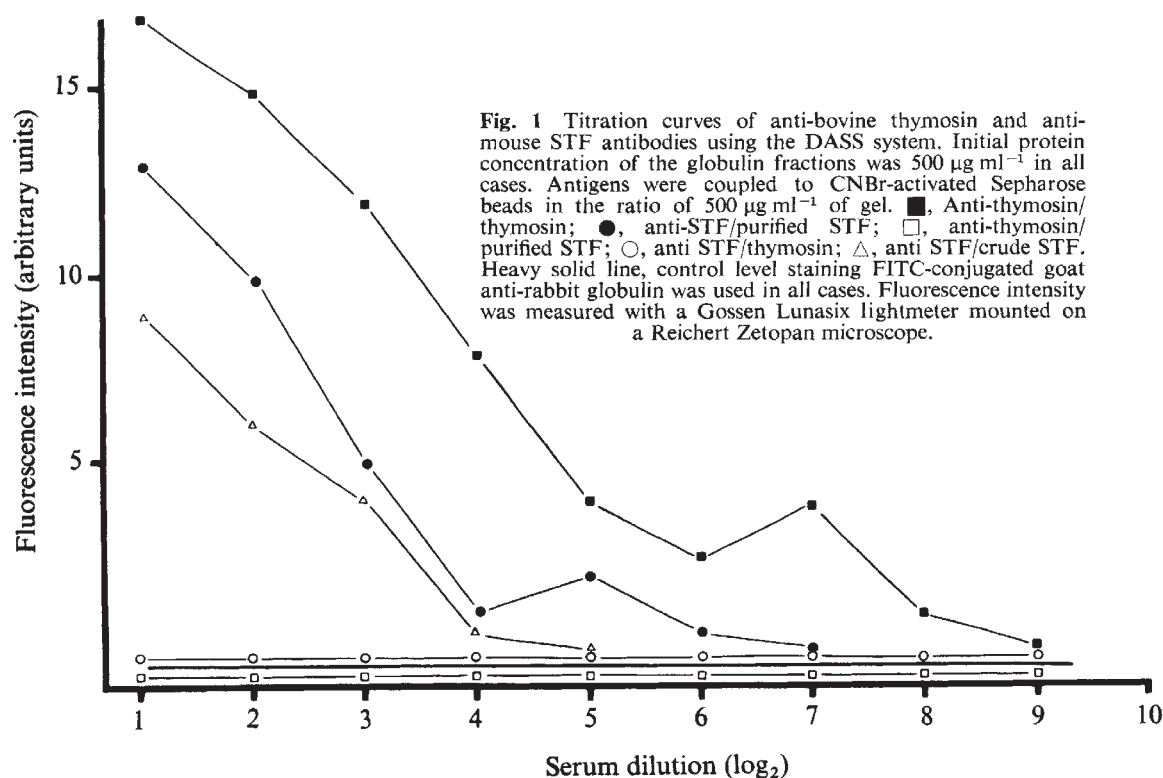
Cellular localisation and antigenic species specificity of thymic factors

It is now generally accepted that T-cell differentiation is influenced by several thymic factors^{1,2} and that thymic reticuloepithelial cells are responsible for the production of these factors. Antibodies to a soluble thymic factor (STF) have been reported to inhibit T-cell differentiation in chicken³ and anti-thymosin globulin has been shown to inhibit the recovery of T cells in ATS-treated mice⁴. Such antibodies therefore can be used to advantage in studies on the modulation of T-cell functions; in this context, a preliminary knowledge of the antigenic species specificity of thymic factors becomes a primary concern.

In this report, we present evidence that bovine thymosin and mouse STF are both found in thymic reticuloepithelial cells of their respective species. Antibodies to these two factors, however, do not cross react.

Anti-STF serum was produced in rabbits according to the technique described previously⁵. Briefly, outbred mice were lethally irradiated and a saline extract was prepared from their lymphocyte-free thymuses (crude STF) which was used for immunisation. The globulin fraction of this antiserum, obtained by ammonium sulphate precipitation⁶, was sequentially absorbed with mouse kidney and lung homogenates, bovine kidney homogenates, mouse and bovine thymocyte suspensions and glutaraldehyde-insolubilised whole serum from thymectomised mice. Additional absorptions were performed with kidney homogenates and thymocyte suspensions of rat, hamster and rhesus monkey, on aliquots used for immunofluorescence on these species. Anti-thymosin globulin was produced and absorbed essentially in the same manner as the anti-STF globulin, but using three weekly booster injections instead of only one, after which a positive precipitin reaction was obtained. Thymosin (1mg) (fraction 5) was used for each injection.

Purified STF was prepared by affinity chromatography of the crude STF preparation used for the immunisation, on



columns² containing the specifically absorbed anti-STF globulin described above.

The specificity of antibodies to mouse STF and to bovine thymosin was established by the defined antigen substrate spheres (DASS) fluorescence technique⁷, with thymosin, crude STF and purified STF as substrates against which antibodies to STF and thymosin were titrated. The titration curves (Fig. 1) show that antibodies to STF reacted with purified STF as well as with crude STF although the reaction with the latter was predictably weaker. No reaction was observed between antibodies to STF and thymosin. On the other hand, antibodies to thymosin reacted strongly and exclusively with thymosin. The same batches of antigen-coupled beads were used for titrations with both antisera and with the control serum.

The localisation of STF in thymic reticuloepithelial cells was demonstrated by the immune peroxidase reaction (Fig. 2). With this technique individual reticuloepithelial cells clearly stood out. Numerous positively stained cytoplasmic granules were evident: whether these correspond to vacuoles containing hormone or whether they were a fixation artefact could not be determined by light microscopy.

The antigenic species specificity of thymosin and STF was studied on frozen sections fixed with cold acetone-ethanol by indirect immunofluorescence with FITC-conjugated goat anti-rabbit globulin⁵. Anti-STF globulin on frozen sections of mouse thymus gave a strong fluorescent staining reaction confined exclusively to the reticuloepithelial network (Table 1). The staining pattern was clearly cytoplasmic and uniform: no vacuoles could be distinguished. Occasionally, a negative nucleus was observed

and cytoplasmic processes could be seen joining other cells. When anti-thymosin globulin was applied to mouse thymus sections, the reaction was almost negative. Non-immune globulin absorbed in the same manner as the immune globulin gave a totally negative reaction. In this negative control, reticuloepithelial cells could be identified by their slight grey-bluish autofluorescence easily distinguishable from the bright green specific fluorescence. Mouse spleen sections and thymocyte smears when stained with all three sera were negative throughout. Sections of rat thymus gave a reaction pattern with anti-mouse STF globulin similar to that in mouse although slightly less intense. Anti-bovine thymosin antibodies gave an almost negative reaction. Hamster thymic reticuloepithelial cells gave a strong reaction when antibodies to mouse STF comparable in intensity to that of rat; there was no reaction with anti-bovine thymosin antibodies. Rhesus monkey thymus reacted only

Fig. 2 Thymus frozen section fixed with 4% paraformaldehyde and treated with purified IgG fraction⁸ of rabbit anti-STF globulin and peroxidase conjugated goat anti-rabbit IgG according to Kuhlmann *et al.*⁹. Note the dark stained reticuloepithelial cells, with their cytoplasmic processes, and the non-staining lymphocytes. ($\times 4,800$).

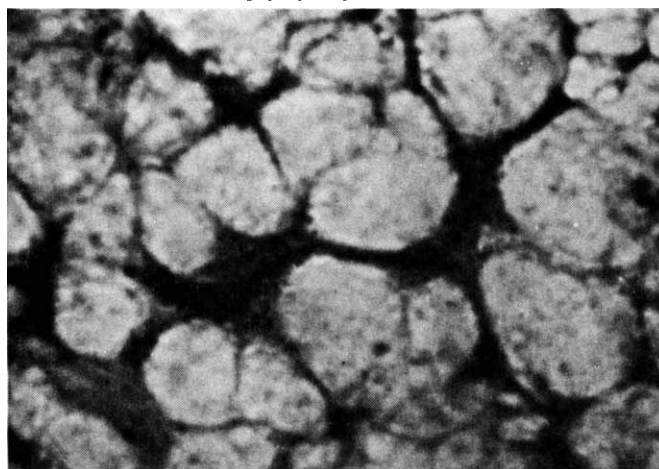


Table 1 Antigenic species specificity of thymic factors

Serum	Immunofluorescent staining of thymic reticuloepithelial cells in				
	Mouse	Rat	Hamster	Monkey	Calf
Anti-mouse STF	++++	+++	+++	(+)	
Anti-thymosin	(+)	(+)			
Non-immune					+++

very slightly with anti-mouse STF antibodies and not at all with anti-bovine thymosin antibodies. Calf thymic reticulo-epithelial cells on the other hand gave a strong reaction with anti-bovine thymosin but no reaction with anti-mouse STF antibodies.

Our results are in agreement with those of Schulof *et al.*¹⁰ who showed by radioimmunoassay that bovine thymosin cross reacted with sheep, horse and goat but not with mouse, rat and human. It would seem that in all species studied by ourselves and others¹¹, thymic hormones are synthesised by reticuloepithelial cells and are antigenically similar among related species. The localisation of thymosin obtained by Mandi and Glant¹² is apparently similar to ours but comparisons are difficult since the species used by those authors are not mentioned.

In conclusion, although the biological activity of thymic factors has been shown not to be species specific¹, their antigenic specificity seems to be restricted to closely related species. Care should therefore be exercised in the choice of species combinations in which studies with antibodies to thymic factors are conducted.

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Generalised deficiency of cytochrome *b₅* reductase in congenital methaemoglobinaemia with mental retardation

RED cells from patients suffering from congenital recessive methaemoglobinaemia are deficient in a reduced nicotinamide dinucleotide (NADH) diaphorase which has a major role in the process of methaemoglobin reduction¹⁻³. This enzyme seems to be identical, or at least related to cytochrome *b₅* reductase (EC 1.6.2.2) for the following reasons: (1) a red-cell-type NADH diaphorase has been found in several different non-erythroid cells from normal human subjects, indicating that it is a widely distributed enzyme⁴; (2) cytochrome *b₅* reductase has been found in the red cells, and cytochrome *b₅* seems to have a direct role in methaemoglobin reduction^{5,6}; (3) in patients with NADH diaphorase deficiency a concomitant deficiency of cytochrome *b₅*

reductase has been observed^{7,8}. In a small but significant proportion of the cases of congenital enzymopenic methaemoglobinaemia, the methaemoglobinaemic syndrome is associated with a progressive neurological disorder with mental retardation causing death at a young age^{3,8,9}. The relationship between methaemoglobinaemia and nerve system involvement has so far been poorly understood, until the finding that the leukocytes of patients with this type of disease also displayed the NADH diaphorase deficiency, whereas nearly normal enzyme levels were found in the leukocytes from methaemoglobinaemic patients without neurological symptoms^{8,10}. It was therefore postulated that methaemoglobinaemia with neurological involvement might result from a generalised enzyme defect^{8,10}.

Here we demonstrate that this is indeed the case by finding a lack of both NADH diaphorase and cytochrome *b₅* reductase in several tissues: red cells, leukocytes, muscle, liver and fibroblasts from a patient suffering from methaemoglobinaemia with mental retardation.

The clinical pattern displayed by the propositus, a 1-year-old female child, was very similar to that previously reported in other cases of methaemoglobinaemia with mental retardation^{8,9}. There was no detectable methaemoglobin reductase activity¹¹ in the red cells. Traces of enzyme retaining a normal mobility were, however, detected on electrophoresis of a concentrated haemolysate followed by specific staining for NADH diaphorase¹². The level of enzyme activity in the red cells of both parents was in the heterozygous range: father: 2.5 U per g haemoglobin (Hb); mother: 2.4 U per g Hb (normal controls: 4.5±0.8 U per g Hb; 1 U corresponds to 1 μmol ferrocyanide-methaemoglobin complex reduced per min (ref. 11)).

To verify the hypothesis of a generalised enzyme defect in this disease, several tissues were investigated. The leukocytes were prepared by PVP sedimentation followed by osmotic shock. Muscle and liver specimens were obtained by surgical biopsy from the propositus, and from a patient of the same age suffering from another condition. Skin fibroblasts were cultured in Eagle's MEM containing 10% calf serum. Cells were collected on day 3 after three passages. All tissue fragments and the pellets of leukocytes and fibroblasts were frozen in dry ice immediately after collection, and stored at -70 °C. The homogenates were prepared with a Potter-Elvehjem apparatus in a 10 mM potassium phosphate buffer, pH 7.5, and sodium deoxycholate (0.5% final) was subsequently added. In the case of leukocytes and fibroblasts, the pellet was pretreated by repeated freezing and thawing before homogenisation. The

Table 1 NADH diaphorase (methaemoglobin reductase) and cytochrome *b₅* reductase activities in several tissues of the propositus

Tissues	Methaemoglobin reductase		Cytochrome <i>b₅</i> reductase	
	Propositus	Control	Propositus	Control
Red cells (Hb-free extract)	0	144	< 0.05	2.0
Leukocytes	2.3	90	< 0.05	2.9
Muscle	6.0	64	< 0.05	2.4
Liver	5.0	94	< 0.05	3.6

Leukocyte, muscle and liver extracts were prepared as described in the text. Hb-free red cell extracts were prepared by DEAE-Sephadex treatment¹³. Methaemoglobin reductase activity was assayed according to Hegesh *et al.*¹¹, and expressed as μmol ferrocyanide-methaemoglobin complex reduced per min per mg protein. Cytochrome *b₅* was prepared from liver microsomes according to Omura and Takesue¹⁴. Cytochrome *b₅* reductase activity was assayed according to Mihara and Sato¹⁵. The reduction of 10 μM cytochrome *b₅* was followed at 556 nm in the presence of 180 μM NADH, and the activity expressed as the first-order rate constant (min⁻¹) per mg protein. Several other enzymes chosen as internal standards were also measured: 6-phosphogluconate dehydrogenase in the Hb-free red-cell extracts; aldolase, hexokinase, phosphoglucomutase, glucose-6-phosphatase and ten lysosomal enzymes in the liver extracts. They were all in the normal range (data not shown).

assays of methaemoglobin reductase activity¹¹ and of cytochrome *b*₅ reductase activity¹⁵ were carried out on the 30,000g supernatant.

From the results summarised in Table 1, it is clear that both enzyme activities are lacking in the propositus, not only in red-cell extracts, but also in leukocytes, muscle and liver. The enzyme defect could also be found in the patient's cultured fibroblasts (Table 2), suggesting that antenatal diagnosis might be possible. To check this possibility, we investigated the cytochrome *b*₅ reductase activity in control cultured amniotic cells. Our results indicate that these cells possess an easily measurable activity, comparable with that found in normal fibroblasts derived from skin (Table 2). The diagnosis of homozygous deficiency should therefore be feasible in cultured amniotic cells.

Table 2 Cytochrome *b*₅ reductase activity in cultured fibroblasts

Cells	Cytochrome <i>b</i> ₅ reductase activity
Skin fibroblasts from the propositus	< 0.05
Skin fibroblasts from control subjects	4
Control cultured amniotic cells	10
	8

Enzyme activity measured and expressed as described in Table 1. Mode of extraction as described in text.

These results bring the first experimental evidence for a generalised enzyme defect in congenital methaemoglobinemia with neurological involvement. In addition they provide further indication that NADH diaphorase (so-called methaemoglobin reductase) and cytochrome *b*₅ reductase are probably identical entities, since both activities were found to be defective in various tissues. This is consistent with previous observations made in normal tissues⁴, and in red cells from normal^{5,6} and methaemoglobinemic subjects^{7,8}. Another possibility is that the two enzymes share a common subunit.

The precise role of cytochrome *b*₅ reductase is still not established. In the red cells, where it is present in a soluble form, this enzyme would act as a methaemoglobin reductase^{5,6}. Elsewhere it is mainly located in microsomes, and has been particularly studied in liver¹⁶ and in brain¹⁷. A soluble form has also been described in rabbit liver cytosol¹⁸. The relationship between the microsomal and the soluble enzyme is not known. Our results now indicate, however, that they are controlled by the same gene since both forms are defective in the propositus: the soluble form in the red cells, the particulate form in deoxycholate-treated liver, muscle and leukocyte extracts. This is in agreement with the immunological relationship that we have found between the cytosolic and the microsomal cytochrome *b*₅ reductase (A.L. and J.-C.K., unpublished). A generalised deficiency of cytochrome *b*₅ reductase is expected to produce a systemic impairment of the metabolic processes linked to cytochrome *b*₅, such as fatty acid desaturation¹⁹. Since the major impact of the disease seems to be on the central nervous system, it would be very important to analyse the fatty acid composition of brain lipids in such patients.

Thus the molecular basis of congenital methaemoglobinemia seems to be a defect of the soluble cytochrome *b*₅ reductase in the form without brain involvement, expressing as a red-cell disease, and a combined defect of both the cytosolic and the microsomal cytochrome *b*₅ reductase in the form with mental retardation, manifesting as a generalised disease.

The explanation for the two types of cytochrome *b*₅ reductase deficiency is still speculative. If the enzyme is coded by a single gene, several hypotheses can be made: (1) the abnormal gene product is unstable, but produced at a normal rate, and only mature red cells, which cannot synthesise proteins, are affected; if the mutation causes

drastic instability or underproduction of the enzyme, the defect is generalised; (2) some mutations only impair the formation of the soluble enzyme from a microsomal precursor, and the enzyme defect is only expressed in the red cells, whereas other mutations produce a defect of both the microsomal and the soluble forms of enzyme, thus causing a generalised disease. If two genes are implicated, one can postulate that methaemoglobin reductase and cytochrome *b*₅ reductase share a common subunit which is defective in the generalised disease.

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Normal spermatozoa from androgen-resistant germ cells of chimaeric mice and the role of androgen in spermatogenesis

IN androgen-resistant male mice of genotype *Tfm/Y* spermatogenesis is arrested at first meiotic division. This is likely to be due, at least in part, to the need for testosterone if spermatogenesis is to proceed beyond prophase of first meiosis¹. It would be somewhat surprising if the response to androgen involved any gene action in the developing sperm cell itself, since the locus of the gene for response to androgen, *Tfm*, is on the X chromosome². The X-Y chromosome pair is believed to become genetically inactive during meiotic prophase³, and at stages beyond meiosis I only 50% of spermatocytes and spermatids possess an X chromosome.

To test this point we made male mice chimaeric for androgen-resistant (*Tfm/Y*) and normal (+/Y) genotype by the technique of embryo aggregation^{4,5}, and tested their fertility to determine whether spermatozoa were formed from the *Tfm/Y* component and were able to take part in normal fertilisation. Of 57 visibly chimaeric animals produced, 2 fertile males proved to be of the correct *Tfm/Y* ↔ +/Y genotype and both produced numerous offspring from their *Tfm/Y* component, indicating that the requirement for testosterone in spermatogenesis does not involve the cell-autonomous action of the *Tfm* locus in the germ cells.

In two series of experiments embryos from matings of *Tfm*+/*+**Ta* × *Ta*/Y were fused with homozygous pink-eye (*pp*) or albino (*cc*) embryos, chosen so that the coat colour variegation in the resulting chimaeras would give some visual indication of the proportions of the two components, at least in the pigment cells. The albino stock also carried the chromosome marker *Rb* 1A1d. The gene *tabby* (*Ta*), which is closely linked to *Tfm*, was used as a marker in the *Tfm* stock.

The *Tfm* component of any animal could be of four possible types: *Tfm*+/*+**Ta*, +*Ta*/+*Ta*, *Tfm*/Y or +/Y (if the rare crossovers between *Tfm* and *Ta* are neglected), whereas the other component could be XX or XY. The genotype of any particular animal was established by a combination of visual inspection and breeding tests. The presence of the *Ta* gene might be detected in the coat of animals with *Tfm*+/*+**Ta*, +*Ta*/+*Ta* or *Ta*/Y components, and some evidence of resistance to androgen might be seen in male animals carrying either *Tfm*+/*+**Ta* or *Tfm*/Y components. Drews *et al.*⁶ have described the intersexuality of chromosomally XX animals, heterozygous for the *Tfm* gene, and sex-reversed to male by the gene *Sxr*, and, since chimaeric animals commonly show phenotypes somewhat similar to those of heterozygotes for X-linked genes⁷, we expected to observe intersexuality of some kind in our chimaeras.

Table 1 Animals produced from two series of embryo aggregations

	Series 1 (<i>pp</i>)			Series 2 (<i>cc</i>)		
	♀	♂	<i>Tfm</i> /Y	♀	♂	<i>Tfm</i> /Y
Non-chimaeric	9	12	8	3	1	—
	♀	♂	Intersex	♀	♂	Intersex
Chimaeric						
<i>pp</i> or <i>cc</i> only*	3	—	2	10	6	6
<i>Ta</i> only	5	3	—	—	—	—
<i>Ta</i> and <i>pp</i> or <i>cc</i>	4	7	—	5	4	2

Matings of *Tfm*+/*+**Ta*♀ × *Ta*/Y♂ and of PT♀ (genetically *aa bb cc^{ch} cc^{ch} dd pp ss sese*) by *pp*♂ (or in the second series of *cc Rb* 1A1d *Rb* 1A1d ♀ by *cc Rb* 1A1d *Rb* 1A1d ♂) were detected by inspection for vaginal plugs. Two days later the eggs (at the four to eight-cell stage) were flushed from the oviducts into medium by the usual techniques⁸ and the zona pellucidae were removed by treatment with pronase. One egg of each type—*Tfm* and *pp* or *cc*—was introduced into a drop of medium under oil, and the two eggs were pushed together. The eggs were then cultured overnight at 37 °C. The following day the eggs were washed in medium and those apparently viable embryos in which good fusion had occurred were transferred to the uterus of a pseudopregnant recipient female (of C3H/HeH × 101/H hybrid type) which had shown a vaginal plug by a sterile male 2 d previously. Usually, only one uterine horn of each female was injected, with four or five embryos. *Visible variegation in coat.

We did indeed find 10 animals with some degree of intersexuality of the genitalia (Table 1), ranging from a mainly female phenotype with masculinised clitoris, through various degrees of ambiguity, to a mainly male phenotype with small teats present. If as expected these animals had a *Tfm*/Y or *Tfm*+/*+**Ta* component there should be little evidence of the *Ta* gene in the coat, and indeed only two of the 10 showed slight expression of *Ta*, whereas 14 out of 20 true males showed *Ta*. The six intersexes judged to be sufficiently masculinised to be potentially able to mate as males were placed with *pp* or *cc* females and these females were inspected for vaginal plugs daily. On this criterion three males mated repeatedly and of these two, both from the *cc* series, proved to be fertile. One was only barely fertile, and has so far produced only 16 offspring in five litters, all albino, that is from his *cc* component. Chromosome tests on cultures of ear skin showed him to have XX and *Rb* 1A1d XY cell lines and he was therefore presumed to be *Tfm*+/*+**Ta* ↔ *cc* XY, although no *Ta* stripes were visible in his coat. The other was fully fertile and produced young from both his components, that is 21 pigmented and 45 albino. The pigmented offspring were all non-*Ta*, and all of four females genetically tested proved to be *Tfm*/+. This is as expected if the chimaeric parent's pigmented component was *Tfm*/Y, and

since he produced both sexes of offspring from both cell lines this male was proved to be of the genotype *Tfm*/Y ↔ *cc* XY. In addition, all the phenotypically normal males with no signs of intersexuality were bred from and all proved fertile. Thirteen produced young from their pigmented component and in all cases except one all the daughters were *Ta*/+ (that is the pigmented component was *Ta*/Y). The one remaining animal, again from the *cc* series, with no intersexuality and no sign of *Ta* in his coat, produced 26 pigmented young, all non-*Ta*, and 27 albino. All five pigmented daughters which were genetically tested proved to be *Tfm*/+, that is this chimaeric male also was proved to be *Tfm*/Y ↔ *cc* XY.

In the first of the two fertile *Tfm*/Y ↔ *cc* XY chimaeras the *cc* component apparently predominated, since his coat was at least 95% *cc*, he was fully masculinised externally (that is of the non-*Tfm* type) except for a pair of small abdominal teats, and his offspring (as already mentioned) were about 67% *cc* and 33% *Tfm*. In the other animal, however, as much as 50% of the coat and of the offspring were of the *Tfm* type, although as previously mentioned he was fully masculinised externally.

Thus, these two males both provide evidence that male germ cells genetically resistant to testosterone through the presence of the *Tfm* gene are capable of normal maturation and fertilisation—the requirement for testosterone in spermatogenesis does not involve the cell-autonomous action of the normal allele of the *Tfm* locus in male germ cells.

Various possible mechanisms for the normal maturation of *Tfm*/Y sperm in a chimaeric testis with genetically normal cells present can be envisaged. The first is that the *Tfm* locus is not involved in the response of the seminiferous epithelium to androgen, and that the arrest of spermatogenesis in *Tfm*/Y non-chimaeric animals is due to cryptorchidism or other secondary factors. This seems unlikely as the *Tfm* locus has been shown to be involved in almost all other responses to testosterone^{9,10}.

Another possibility is that the *Tfm* locus is involved but acts not in the germ cell, but in another cell type, for example the Sertoli cell or the tubular epithelial cells. The role of the Sertoli cells in the maintenance of spermatogenesis by testosterone is well established. One product of the Sertoli cells is an androgen binding protein (ABP) secreted into the lumen of the seminiferous tubules¹¹. Clearly in a chimaera such a free protein would be available to germ cells of both components. Although ABP is androgen-dependent¹² it is not, however, clear that it is involved in spermatogenesis or that it is determined by the *Tfm* locus, which is known to control a cytosol androgen-receptor^{13–15}. Such a receptor is present in the seminiferous tubules, but Hansson *et al.*¹⁶ could not establish whether it was produced by the Sertoli cells or the germ cells.

Thus, there is the third possibility that in the *Tfm*/Y ↔ +Y chimaeras the relevant protein could be passed from one germ-cell type to the other. This seems unlikely in view of the high fertility and relatively high proportion of *Tfm*-type young produced by our two males. Although it is known that intercellular bridges occur normally both among spermatogonia¹⁷ and in spermatids, these are presumably between cells descended from a single stem cell and therefore genetically from the same component of a chimaera. There is no reason to suppose that cooperation between developing sperm from different stem cells could occur to a sufficient degree to rescue 50% of the fertilising sperm, as in our second animal. On the other hand, derivation of the necessary protein from Sertoli cells or other tubule cells seems much more plausible. Since these cells are derived embryogenically from a different cell population, *Tfm*/Y germ cells may easily have been in close contact with +/Y Sertoli cells and moreover the proportion of the +/Y component in the Sertoli cells of the two fertile chimaeras may well have been higher than in the germ cells. Further information may be obtainable later from histological sections of the testes, but at present the mice are needed for breeding homozygous androgen-resistant daughters.

Thus, these data support but do not conclusively prove that the response to androgen of the seminiferous epithelium is entirely mediated by testicular somatic cells. Similarly, testosterone is required for normal maturation of spermatozoa in the epididymis^{18,19}, and our data again indicate that action of the *Tfm* locus in the spermatozoa themselves is not involved. If, as seems likely from Drews and Alonso-Lozano's²⁰ study of the epididymis in *Tfm/+ Sxr/+*, gene action at this locus in the epididymal cells is necessary, then clearly in a chimaera a sufficient proportion of *+/Y* cells can ensure maturation of spermatozoa from both components.

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Electrical properties of 'dark green' and 'yellow' neurosecretory cells in the snail *Lymnaea stagnalis* L.

A NUMBER of electrophysiological investigations of neurosecretory neurones have been carried out in gastropod molluscs¹⁻⁶ but in every case except one⁷ the neurosecretory status and function of the cells was uncertain. In the pulmonate snail *Lymnaea stagnalis* L., substantial morphological and histochemical evidence obtained by light and electron microscopy exists for regarding the 'dark green' (DGCs) and 'yellow' (YCs) neurones as neurosecretory⁸⁻¹⁰. The distinction between DGCs and YCs was based on their colour reaction to a combined Alcian blue-Alcian yellow staining technique used on sections of fixed brain material viewed under the light microscope⁷, but differences in elementary neurosecretory granule diameter and axon terminal location have also been found^{7,8}. Good evidence also exists for a function of DGCs and YCs in ionic or osmotic regulation in *Lymnaea*; the rate of release of both DGC and YC neuroendocrine material increased when snails were kept in deionised water and decreased in hypertonic saline⁸⁻¹⁰. We show here that it is possible to identify DGCs and YCs in electrophysiological experiments and characterise them on the basis of their location, appearance and electrical properties in the live brain.

Large specimens of commercially supplied *Lymnaea stagnalis* (2.9-6.2 g) were kept in Brighton tapwater and fed on lettuce. Electrophysiological experiments were carried out on isolated brains (from 83 animals) immersed in blood just previously collected from the intact snail. Some dissection of loose connective tissue surrounding the brain was often necessary to see the superficially situated neuronal

cell bodies, and the inner sheath surrounding the brain was treated for short periods with Protease (Sigma, Type V) to aid intracellular penetration of neuronal cell bodies with microelectrodes. Electrical signals were recorded intracellularly from neuronal cell bodies and fed into a Bioelectric Instruments amplifier (Farmingdale, USA) which allows bridge balance of electrode resistance and current injection through the recording electrode while recording resulting changes in membrane potential. Electrodes were filled with 2 M K₂SO₄ or 6% Procion Yellow M-4RS. Recorded potentials were displayed on a storage oscilloscope and permanently recorded on film or pen recorder.

To identify the neurones from which recordings had been made as YCs or DGCs, their colour reaction to Alcian blue-Alcian yellow (AB-AY) treatment in histological preparations was investigated. Candidate neurones were marked with Procion Yellow by iontophoresis from the recording electrode in the conventional manner¹¹; only one cell per ganglion was injected. The brain was fixed in Stieve's fluid, embedded in wax, serially sectioned, and the sections mounted on slides without coverslipping. The marked cells were found under ultraviolet light in the light microscope, photographed and the cell body locations carefully noted. The sections were then stained by the AB-AY method⁷ and the staining reaction of the cells recorded.

Dark green cells were found on the ventral surface of the two pleural ganglia where, from morphological studies, they are known to occur⁷. They were 30-50 μ m in soma diameter *in vivo*, and their orange colour was often similar to the surrounding non-neurosecretory neurones¹². In some preparations they were orange with a slight white tint in reflected white light, but never the white or blue-white of other *Lymnaea* neurosecretory cells¹³. Twenty-nine DGC were marked with Procion dye and subsequently confirmed by AB-AY staining.

DGCs always showed low frequency steady firing (Fig. 1a) at mean rates within the range 10-60 action potentials per min. Spike shape was conventional, with duration 12-20 ms (at 50% spike height) with spikes showing a pronounced after-hyperpolarisation (Fig. 1b). Spike shape and firing pattern were quite different from YCs (Fig. 2). Depolarising postsynaptic potentials were seen in DGCs when the cells were hyperpolarised (Fig. 1c-e). Their amplitude was increased by hyperpolarisation and as they did not reverse polarity at threshold for impulse initiation,

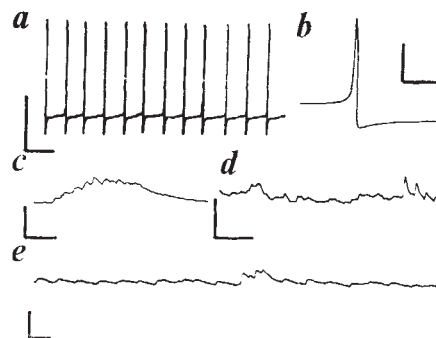


Fig. 1 Electrical properties of 'dark green' neurosecretory cells from *Lymnaea*. *a*, Example of low frequency spike activity recorded in all DGCs; *b*, DGC action potential on fast time base to show impulse shape with its prominent after-hyperpolarisation; *c-e*, e.p.s.p.s recorded in DGC whose spike activity was suppressed by application of steady hyperpolarising current. *c*, Compound e.p.s.p. of long duration, about 10 s. *d*, Continuous high frequency e.p.s.p.s. input to DGCs causes irregular baseline fluctuations where individual e.p.s.p.s are difficult to distinguish except for two unitary large e.p.s.p.s (largest one, 10 mV) towards the end of the trace. *e*, Continuous e.p.s.p.s of low amplitude (1-2 mV) and around 500 ms duration typical of those recorded in most DGCs. Occasional larger e.p.s.p.s are imposed on this basic input. Scale bars: *a*, 2 s, 40 mV; *b*, 100 ms, 40 mV; *c*, 2 s, 10 mV; *d*, 1 s, 10 mV; *e*, 1 s, 5 mV.

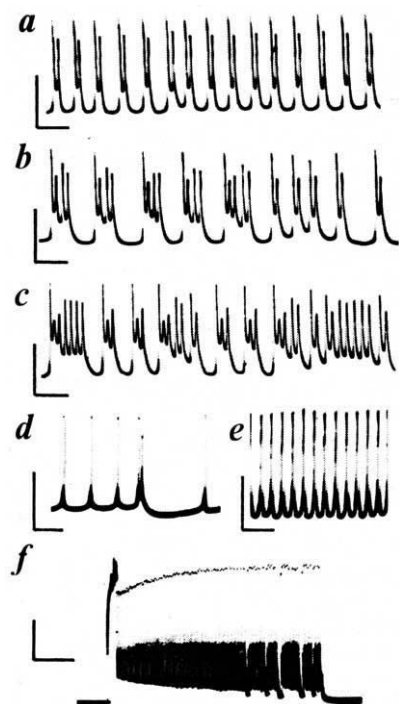


Fig. 2 Intracellular recordings from 'yellow' neurosecretory cells from *Lymnaea*. *a-c*, Spontaneous firing of a YC from the ventromedial surface of the right pleural ganglion. The cell was gradually depolarising so that in *a* doublet spikes were predominant, whereas at higher levels of depolarisation grouped firing of mixed doublets and triplets (*b*) were seen which changed to longer duration burst at the highest level of depolarisation shown (*c*). YCs at just suprathreshold polarisation for spike initiation often generate single spikes with occasional doublets (*d*). Note the larger hyperpolarisation following the doublet spike compared with the single. At very high levels of depolarisation, YCs show a high frequency continuous single spike pattern (*e*). *f*, An example of a phenomenon seen in a few YCs: the cell was silent but a single depolarising square pulse of current which had to be large enough to generate spikes produced a long self-sustaining burst of spikes lasting about 15 s. Towards the end of the burst doublet spikes were formed. Scale bars: *a-c*, 1 s, 30 mV; *d*, 1 s, 40 mV; *e*, 1 s, 30 mV; *f*, 2 s, 20 mV.

they are chemically mediated excitatory postsynaptic potentials (e.p.s.p.s). One type of e.p.s.p. was continuous in occurrence and of low amplitude (1–3 mV) (Fig. 1*e*), whereas a second type of e.p.s.p. occurred sporadically but was of larger amplitude (4–10 mV) (Fig. 1*d*). Occasionally, large compound e.p.s.p.s were recorded, which presumably resulted from high frequency bursts in presynaptic cells (Fig. 1*c*).

Yellow cells occurred in the ganglionic locations noted by Wendelaar Bonga⁷ but also commonly on the medial surface of the pleural ganglia. They were 50–70 μ m in soma diameter *in vivo*, and, unlike DGCs, were usually clear white in colour or sometimes orange-white when viewed in reflected white light. Their white axons could often be seen traversing the ganglion surface to the connectives and sheath tissue surrounding the brain. Twenty-one marked YCs were identified by AB–AY staining but 40 others were identified on the basis of position, colour and electrical properties, once these were known.

The distinctive feature of YCs is their tendency to generate double spikes (Fig. 2). These either occurred spontaneously or could be generated by setting the cells at the appropriate level of polarisation. The double spikes were best seen when the cell membrane potential was just suprathreshold for impulse initiation (Fig. 2*a*). At higher levels of applied depolarisation, or spontaneously, bursts of spikes were observed which consists of mixed doublets, triplets and single spikes (Fig. 2*b* and *c*). At high levels of depolarisation, the cells fired in a continuous

single spike pattern (Fig. 2*e*). Some YCs when firing at low frequency (less than one spike every few seconds) also generated single spikes with occasional doublets (Fig. 2*d*). An interesting variation in YCs bursting tendency was found in four cells. They were silent, but a single supra-threshold depolarising square pulse of at least 0.5 s duration induced a self-sustaining burst of impulses which lasted up to 45 s (Fig. 2*f*). The burst consisted mostly of single spikes, but a few double spikes formed part of most bursts in a particular cell. No synaptic input was seen which was responsible for the spike activity seen in many YCs, which thus seems to be endogenous in origin.

The results show that the DGCs and YCs are distinct in appearance and electrical properties and we can now recognise these cells *in vivo* without the need for dye marking and subsequent staining.

Roubos⁹ has shown that the secretory responses of DGCs to deionised water and saline solutions can occur in isolated ganglia when these are transplanted into experimental animals. Receptors mediating these responses must therefore lie within the brain, and these may be the source of excitatory input to the DGCs. Previous electron microscopic evidence had not revealed the presence of synapse on DGCs⁸.

The tendency of YCs to fire bursts of spikes of variable height is very distinctive and has not been seen in other molluscan neurosecretory neurones which burst, such as R15 of *Aplysia*⁴ or cell 11 of *Otala*².

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Marked prolongation of post-tetanic potentiation at a transition temperature and its adaptation

A UNITARY, monosynaptic excitatory postsynaptic potential (e.p.s.p.) can be recorded from cell R15 of the abdominal ganglion of *Aplysia californica* on appropriate stimulation of the right visceropleural connective. The amplitude of this e.p.s.p. undergoes a sequence of changes with trains of one or two stimuli per second. These changes include synaptic depression, frequency facilitation and post-tetanic potentiation. As described in detail elsewhere^{1,2}, all these phenomena are apparently due to alterations in the amount of neurotransmitter released rather than to postsynaptic mechanisms. The frequency facilitation, a relatively transient phenomenon, is believed to be limited by the rate of neurotransmitter mobilisation into the pool available for release². In contrast, the post-tetanic potentiation, a much more sustained phenomenon, is believed to be due to a

change in the fraction of the available pool of transmitter released per stimulus².

To evaluate further the relationship of frequency facilitation to post-tetanic potentiation (PTP) we studied the effects of changing the temperature of the preparation. We found that the effects of temperature provided another means for distinguishing the mechanism underlying these phenomena. A more striking finding was the discontinuous relationship between temperature and the duration of PTP. This provided clues to the mechanism of PTP.

The abdominal ganglion of *A. californica* was pinned in a dish, and perfused with modified seawater as described previously^{1,2}. The temperature of the preparation was maintained within $\pm 0.25^\circ\text{C}$ with a thermoelectric module (Melcor) regulated by a thermistor placed close to the ganglion. Cell R15 was hyperpolarised to eliminate endogenous bursting; stimulation and intracellular recording were as described before^{1,2}. Preparations were stimulated at 2 pulses s^{-1} for 100 stimuli. When the train stopped, test pulses were given every 20 s to obtain an approximation of the maximal amplitude of the e.p.s.p. during the PTP period. Thereafter, test pulses were given at 10-min intervals to obtain an estimate of the duration of PTP.

We found that the duration of PTP, which was slightly prolonged by reducing the temperature from the usual 15°C to 12°C , was strikingly prolonged when the tempera-

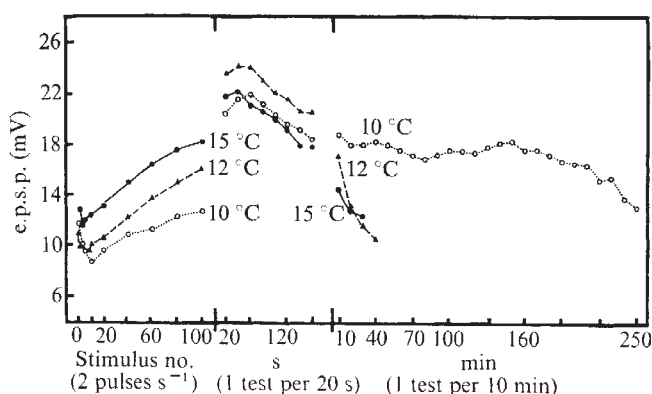


Fig. 1 Amplitude of the unitary and monosynaptic e.p.s.p. obtained in cell R15 on minimal stimulation of the right visceropleural connective. The following stimulus pattern was given sequentially at 15°C , 12°C and 10°C : 100 stimuli at a frequency of 2 s^{-1} followed by eight test pulses at 20-s intervals and then by test pulses every 10 min until the size of the e.p.s.p. had returned to the initial level. The temperature was then lowered and the preparation was allowed to equilibrate for 30 min without stimulation before the stimulus sequence was repeated. Neither the amplitude of the first e.p.s.p. of a train nor the maximum size of the PTP were significantly affected by a change in the temperature. The frequency facilitation during the trains showed a fairly linear temperature dependence, while the duration of the PTP was abruptly prolonged between 12°C and 10°C .

ture was reduced to 10°C (Figs 1 and 2a) or 8°C . The relationship of the duration of PTP to temperature is, therefore, discontinuous in that a transition temperature is observed. In four control experiments in which no trains of stimuli were given we found that the amplitude of an e.p.s.p. elicited every 10 min remained constant when tested for 4 h each at 10°C and 8°C . Therefore, the enlarged e.p.s.p. amplitude observed after a train at 10°C and 8°C is due to prolongation of PTP rather than to temperature effect on an e.p.s.p. elicited every 10 min, as is done when testing the duration of PTP.

In contrast to the findings with PTP duration, the amplitude of frequency facilitation (measured as the ratio of the hundredth e.p.s.p. of the train to the first) showed a relatively smooth dependence on temperature (Fig. 1 and Table 1); the amplitude of the first e.p.s.p. of a train and the maximal amplitude of the e.p.s.p. measured during the PTP were not significantly affected (Table 1).

Since poikilothermic animals such as *Aplysia* show adaptation to changes in ambient temperature³, we determined the effects of maintaining the animals at 11°C rather than the usual 20°C for 2 d before removal of the ganglion. We found that the duration of PTP no longer showed a transition at 10°C or 8°C . In several animals, however, there appeared to be a transition when the temperature was reduced to 6°C (Fig. 2b). We also found that when the isolated ganglion was maintained for 4 h at 10°C before stimulation, the duration of PTP did not show a transition at several low temperatures (Fig. 2c).

Temperature dependence of various neurophysiological phenomena, such as the duration of PTP at neuromuscular junctions^{4,5}, has been reported. The effects of temperature on frequency facilitation that we observed are typical of the temperature dependence of many enzymatic reactions—that is, a linear dependence on temperature over a wide range³. The nonlinear relationship between temperature and duration of PTP is, in contrast, more reminiscent of reactions in systems where activity depends on the state of fluidity of lipids. For example, membrane transport systems that contain lipid and protein components show a striking change in a level of function at transition temperatures⁶⁻⁸. Although transition temperatures in protein function in the absence of lipids are also possible, most known phenomena of the type observed here are due to phase changes in lipids at critical temperatures. The rapid adaptation observed here could be related to known responses to temperature change that could affect protein structure, lipid fluidity or both. Changes in lipid composition of membranes in bacteria, with altered membrane function, have been observed in less than an hour^{6,9}.

These considerations raise the possibility that PTP is due to a change in the state of organisation of a membrane component of the presynaptic nerve terminal; and that the rate of decay of this new state of membrane organisation is strikingly retarded at a temperature at which there is a reduction in fluidity of the lipids associated with this state

Table 1 Effect of temperature on an isolated e.p.s.p. maximal PTP and frequency facilitation

Temperature ($^\circ\text{C}$)	8	10	12	15	20
Isolated e.p.s.p.*	0.94 ± 0.10	0.93 ± 0.06	1.00	1.02 ± 0.06	1.01 ± 0.10
Maximal PTP†	0.93 ± 0.12	1.00 ± 0.05	1.00	0.98 ± 0.05	0.96 ± 0.08
Frequency facilitation‡	0.72 ± 0.06	0.88 ± 0.05	1.00	1.04 ± 0.06	1.12 ± 0.11
No. of preparations	3	10	10	6	3

All values and ratios are expressed as relative to the measurement at 12°C , which is set at 1.00.

*The amplitude of the first e.p.s.p. of a train. None of these groups differed significantly.

†Defined as the ratio of the amplitude of the maximum measured e.p.s.p. during the PTP period to that of the first e.p.s.p. of the train. Results are shown relative to the measurement at 12°C . None of these groups differed significantly.

‡Defined as the ratio of the amplitude of the last e.p.s.p. of the train to the first e.p.s.p. of the train. Results are shown relative to the measurements at 12°C . The groups at 8°C and 10°C differed significantly ($P < 0.05$) from the 12°C group. The groups at 15°C and 20°C differed significantly ($P < 0.025$) from the 10°C group.

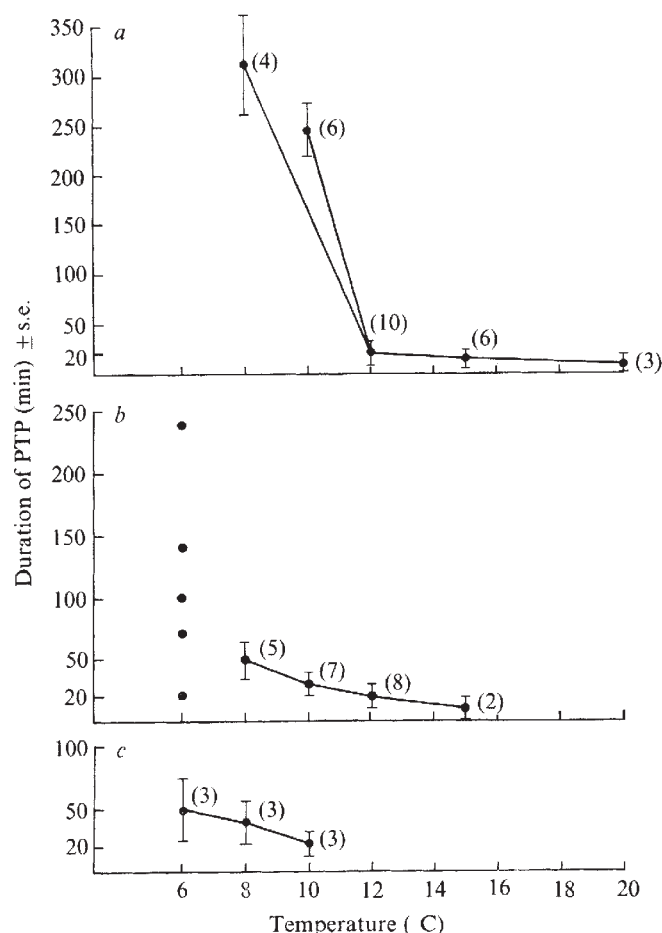


Fig. 2 Dependence of the duration of PTP on temperature. The duration of the PTP was estimated by administering test pulses at 10-min intervals until the size of the e.p.s.p. returned to the size of the first e.p.s.p. of the train (Fig. 1). The number of experiments at each point is shown in parentheses. *a*, Pooled data from 10 preparations. Every preparation was tested at 12 °C. In six preparations, the temperature was then lowered from 12 to 10 °C whereas in four others the temperature was taken from 12 °C directly to 8 °C. *b*, Pooled data from four animals which were maintained at 11 °C rather than the usual 20 °C for 2 d before the experiment. The duration of the PTP was only slightly prolonged when the temperature was lowered from 12 °C to 10 °C or 8 °C but at 6 °C some preparations showed an abrupt prolongation of the PTP. None of the preparations treated in this fashion showed a temperature transition between 12 and 10 °C. *c*, Three isolated ganglia were maintained at 10 °C for 4 h before stimulation. In these conditions the duration of the PTP measured at 10 °C was short compared with control animals (*a*) and no abrupt prolongation occurred when the temperature was lowered to 6 °C.

of membrane organisation. Consistent with this possibility is the finding¹⁰ that ethanol (4%) and butanol (0.1%), agents that increase membrane fluidity^{11,12}, increase the rate of decay of PTP at 15 °C about fivefold. Some presynaptic membrane functions that might be critical to these findings include: (1) regulation of calcium levels in the nerve terminal by calcium uptake functions of mitochondrial membranes¹³; and (2) regulation of fusion of synaptic vesicles with release sites on the plasma membrane through changes in the lipid-protein order of the presynaptic membrane¹⁴. PTP might be due to a change in the state of order of one of these membranes as a result of repetitive stimulation; the decay of PTP might then be retarded by treatments that decrease membrane fluidity (low temperature) and accelerated by treatments that increase membrane fluidity (alcohols).

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Prolongation of hippocampal inhibitory postsynaptic potentials by barbiturates

It is well documented that barbiturates dramatically prolong presynaptic inhibition¹⁻⁴. Their effect on postsynaptic inhibition is less clear, although the available evidence suggests that a similar enhancement may occur at some sites⁵⁻⁷. Since the inhibitory postsynaptic potentials (i.p.s.p.s) in hippocampal neurones are unusually large and the neurones are readily penetrable by microelectrodes⁸, we have studied the effect of barbiturates on these i.p.s.p.s in detail. We have found that barbiturates hyperpolarise hippocampal neurones and markedly prolong the i.p.s.p. by a direct action on inhibitory synapses, anaesthetic doses increasing the duration fivefold. The hyperpolarising action of barbiturates and their effect on i.p.s.p.s would both contribute to the neural depressant action of these anaesthetic agents.

Cats were anaesthetised either with 80% nitrous oxide and 0.3% Halothane or with an intraperitoneal injection of pentobarbitone (35 mg kg⁻¹). The animals were paralysed with Flaxedil and artificially ventilated, the end tidal CO₂ being maintained at 3.5%. A bilateral pneumothorax and cisternal drainage were carried out. Femoral blood pressure was continuously monitored and the femoral vein was cannulated for the administration of barbiturates. After cutting a window in the skull, the hippocampus was exposed by removing the overlying cortex with suction. Intracellular recording was limited to CA₁ neurones. Stimulation with a local surface electrode produced a large i.p.s.p. in these neurones⁸. Immobilisation of the hippocampus was secured by the following procedures. A thin layer of 4% agar in Ringer's solution was placed on the surface of the hippocampus. A conical shaped pressure foot was placed on the agar and the surrounding area was entirely filled with low melting point wax which formed a rigid connection with the bony edges of the skull. The microelectrodes were filled with KCl 2.4 M, potassium methylsulphate 2 M, or potassium citrate 2 M. No difference in the effect of barbiturates on the i.p.s.p. was noted

with these different salts, although the i.p.s.p. was usually depolarising with the KCl-filled electrodes. The electrode resistance varied from 20–50 M Ω . The resting membrane potential and i.p.s.p. size were continuously monitored on a pen recorder and film records were obtained at appropriate intervals.

In the first series of experiments the i.p.s.p.s recorded from a number of neurones during 80% nitrous oxide anaesthesia were compared with the i.p.s.p.s recorded after the animal had been given 30 mg kg⁻¹ pentobarbitone intravenously and the nitrous oxide had been turned off. The activity of neurones in the latter group was recorded within 2 h after the pentobarbitone injection, only neurones having membrane potentials greater than -35 mV being used. With nitrous oxide anaesthesia the mean half-decay time of the i.p.s.p.s was 26.8 ms $\pm$ 10.2 s.d. ($n=33$), whereas with pentobarbitone anaesthesia it was 128.3 ms $\pm$ 28.4 s.d. ($n=24$). The degree of prolongation was independent of the resting membrane potential. These results show that in the presence of pentobarbitone the i.p.s.p. is prolonged greatly.

To examine these effects in more detail, barbiturate was injected while recording from neurones. The results are based on 29 neurones. With eight of these neurones the i.p.s.p.s were recorded during nitrous oxide anaesthesia and subsequently after barbiturate was injected. In the remainder, the control recording was in a pentobarbitone-anaesthetised animal and the effect of a supplemental injection was examined. The results from these two groups of neurones were similar although, as expected, less dramatic in the latter group. In 16 neurones the recording conditions were so stable that it was possible to examine the effect of barbiturate on the resting membrane potential. In five neurones barbiturate injection effected no change in

membrane potential. In the other 11 neurones the injection resulted in a slowly developing hyperpolarisation, averaging 5.6 mV $\pm$ 2.1 s.d. which was independent of the initial resting membrane potential. The dose of pentobarbitone injected in these experiments varied from 5 to 33 mg kg⁻¹.

The intravenous injection of pentobarbitone produced a dose-dependent prolongation of the i.p.s.p. which was independent of any change in the membrane potential. Figure 1a shows the typical action of pentobarbitone on the membrane potential and i.p.s.p. of a hippocampal neurone. The membrane potential increased from 50 to 60 mV. The amplitude of the i.p.s.p. increased twofold while the half-decay time of the i.p.s.p. gradually increased from 38 to 160 ms. Although a fall in membrane potential occurred at 37 min, presumably due to partial dislodgement of the electrode, there was only a slight transient change in the duration of the i.p.s.p. In Fig. 1b, 15 mg kg⁻¹ pentobarbitone increased the half-decay time of the i.p.s.p. from 27 to 60 ms without any change in membrane potential. The minimum effective dose of pentobarbitone for this effect was 5 mg kg⁻¹. The relative increase in the half-decay time for approximately 10 mg kg⁻¹ had a mean value of 2.15 times $\pm$ 0.7 s.d. ($n=16$). Thiopental (Fig. 1c) and thiamylal (not illustrated) also prolonged the i.p.s.p. A similar lengthening was seen on i.p.s.p.s which had been reversed by diffusion of chloride ions from the microelectrode (Fig. 1d and e). The results on reversed i.p.s.p.s were obtained after the i.p.s.p. had stabilised; therefore, the prolongation cannot be attributed to chloride ions diffusing to more distant inhibitory synapses. In Fig. 1e, the stimulus strength was just above threshold and numerous reversed spontaneous unitary i.p.s.p.s can be seen, particularly in the background responses of *f*. Pentobarbitone (10 mg kg⁻¹) prolonged both the evoked and spontaneous unitary i.p.s.p.s in *e*₂ and *f*₂.

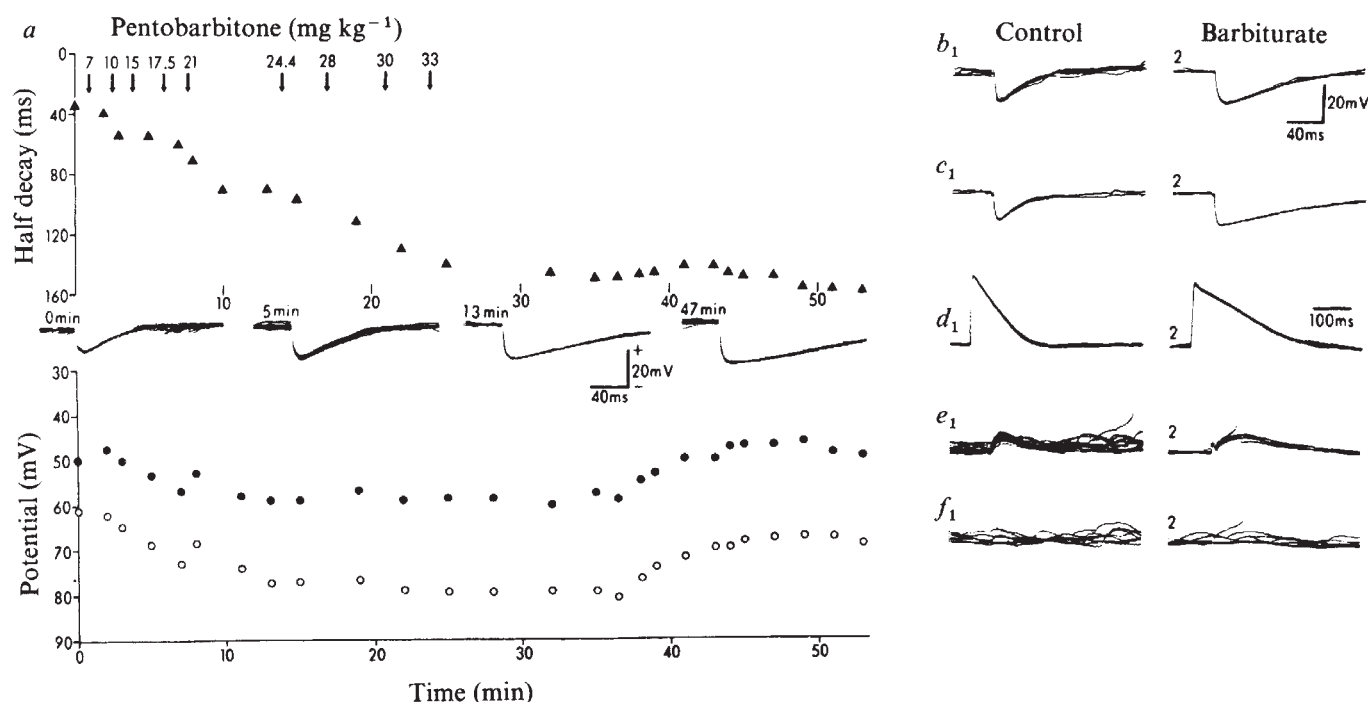


Fig. 1 Prolongation of i.p.s.p.s by barbiturates. *a*, Top graph half decay of the i.p.s.p. (▲); bottom graph membrane potential (●) and peak of the i.p.s.p. (○). The time scale applies to both graphs. Sample film records, taken at the indicated times are shown between the graphs. The cumulative dose (mg kg⁻¹) of pentobarbitone after each injection is shown at top of figure. In *b*, 15 mg kg⁻¹ pentobarbitone was injected into a nitrous oxide-anaesthetised cat. The membrane potential remained stable at -37 mV and *b*₂ was obtained 5 min after *b*₁. In *c*, 17 mg kg⁻¹ thiopental was injected into a nitrous oxide-anaesthetised cat. The membrane potential (-50 mV) remained stable over the 12-min period between *c*₁ and *c*₂. The i.p.s.p. in *d* was depolarising due to diffusion of chloride ions from the microelectrode with the consequent high level of intracellular chloride. Pentobarbitone 10 mg kg⁻¹ was injected into a pentobarbitone-anaesthetised cat. The membrane potential (-55 mV) was constant during the time (8 min) between *d*₁ and *d*₂. The i.p.s.p. in *e* was also reversed by diffusion of chloride ions from the microelectrode. The background recordings, *f*, from this same neurone show numerous reversed unitary i.p.s.p.s. Pentobarbitone 10 mg kg⁻¹ was injected into a pentobarbitone-anaesthetised cat and membrane potential was increased from -43 to -45 mV. In *e*₂ an action potential was elicited from one of the i.p.s.p.s after pentobarbitone. The time between the two responses was 4 min. The time and voltage calibration in *b* applies to all records except for the time calibration in *d*.

The hyperpolarising action of barbiturates on many hippocampal neurones might arise from a number of factors including withdrawal of background excitation⁹, blockage of intracellular oxidative metabolism¹⁰ and activation of γ -aminobutyric acid (GABA) receptors¹¹.

In a previous study on field potentials in the olfactory bulb⁷, it was proposed that barbiturates prolonged post-synaptic inhibition. The present intracellular study on hippocampal neurones provides direct evidence that this is indeed the case. The effects of barbiturates on post- and pre-synaptic inhibition are qualitatively and quantitatively similar. Interestingly, GABA is thought to be the transmitter involved both in pre-^{2,12,13} and in postsynaptic inhibition at supraspinal sites^{14,15}, including the hippocampus^{16,17}. This barbiturate effect could result from prolonged release of inhibitory transmitter, a direct action on the GABA conductance mechanism or delayed removal from postsynaptic receptor sites. The observation that pentobarbitone can prolong the action of directly applied GABA^{11,18} would suggest that prolonged release is not the sole basis for the barbiturate effect. The finding that pentobarbitone is a competitive blocker of GABA uptake¹⁹ suggests that the prolongation may result from delayed removal from postsynaptic receptor sites.

In the past, emphasis has been placed on the depressant action of barbiturates on excitatory synaptic transmission²⁰⁻²⁴. If the dramatic prolongation of hippocampal i.p.s.p.s by anaesthetic doses of barbiturate (approximately four to five times) applies to other inhibitory synapses of the cerebral cortex, a general depression of the excitability of the cerebral hemispheres would be expected. The prolongation of the unitary postsynaptic inhibitions would lead to more effective summation of repetitive inhibitory actions and so account for, at least in part, the action of barbiturates in producing unconsciousness.

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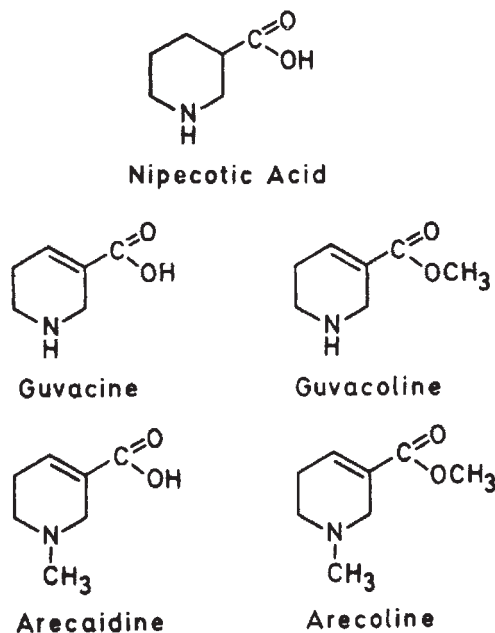
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Betel nut constituents as inhibitors of γ -aminobutyric acid uptake

THE pharmacological properties of betel nut, the dried seed of *Areca catechu* which is consumed in immense quantities in the East as a masticatory, are usually interpreted in terms of the cholinomimetic effects of the major alkaloid component, arecoline. Betel nut contains lesser amounts of the related alkaloid guvacoline and the amino acids arecaidine and guvacine. According to popular tradition, a mixture of crushed betel nut and lime is enveloped in leaves of *Piper betle* before chewing. This treatment with lime has been shown to hydrolyse almost quantitatively arecoline to arecaidine¹, a well tolerated substance which lacks the typical parasympathomimetic effects of arecoline, including tremor and salivation^{1,2}. Studies on the effects of arecaidine on the behaviour of mice indicate that this substance participates in producing some of the psychic changes ascribed to the betel mixture^{1,2}. These betel constituents are tetrahydropyridine derivatives (Fig. 1) and we have shown that nipecotic acid, 'hexahydropyridine', is a potent inhibitor of the uptake of the central inhibitory transmitter γ -aminobutyric acid (GABA) in rat brain slices³. Nipecotic acid potentiates the depressant action of microelectrophoretically administered GABA on the firing of feline spinal neurones⁴. As the uptake of GABA is likely to be concerned with the inactivation of this transmitter, interference with GABA uptake could result in behavioural changes. The structural similarities between nipecotic acid and the betel nut constituents together with the above mentioned findings prompted us to examine the influence of these compounds on GABA uptake. We have found that arecaidine and guvacine are competitive inhibitors of GABA uptake in rat brain slices and we propose that some of the psychic effects of betel nut consumption may be the result of inhibition of GABA uptake.

The 'high affinity' uptake of GABA was studied in slices of rat cerebral cortex using the procedure developed by Iversen and Neal⁵. The high affinity uptake of L-glutamate, glycine, and L-proline was studied in a similar manner⁶⁻⁸. Arecaidine was prepared from commercially

Fig. 1 Structures of nipecotic acid, guvacine, guvacoline, arecaidine and arecoline.



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available arecoline by acidic hydrolysis⁹. Guvacine was prepared as described by McElvain and Stork¹⁰, and guvacoline was prepared from this by esterification according to Freudenberg¹¹. The four compounds were analytically pure, and their infrared and proton magnetic resonance spectra were consistent with the structures in Fig. 1.

Arecoline and guvacoline (0.5 mM) had no effect on the uptake of GABA or L-glutamate. Arecaidine and guvacine (0.5 mM) inhibited the uptake of GABA by 75 ± 3 and $98 \pm 1\%$ (mean $\pm$ s.e.), respectively, but did not inhibit the uptake of L-glutamate, glycine or L-proline. Probit analysis of the results of experiments with appropriate concentrations of arecaidine and guvacine indicated that 50% inhibition of GABA uptake was obtained with $122 \pm 12 \mu\text{M}$ arecaidine and $8 \pm 1 \mu\text{M}$ guvacine. On this basis, guvacine was as potent as nipecotic acid³, which inhibited GABA uptake by 50% at $9 \pm 1 \mu\text{M}$.

All of these experiments were carried out with pre-incubation of the inhibitor with the brain slices for 15 min before addition of radioactive GABA. As has been observed with nipecotic acid¹² and 2,4-diaminobutyric acid¹³, less apparent inhibition of GABA uptake was obtained if arecaidine or guvacine were added at the same time as GABA. This may be interpreted on the basis of these inhibitors being substrates for the GABA uptake system and entering intracellular compartments during the pre-incubation period. Kinetic studies showed that arecaidine and guvacine act as competitive inhibitors of GABA uptake when added at the same time as GABA. The apparent slope inhibition constants (K_{is}) were $141 \pm 38 \mu\text{M}$ for arecaidine and $14 \pm 4 \mu\text{M}$ for guvacine. On this basis, guvacine has a similar affinity for the GABA carrier to nipecotic acid³ which has a K_{is} of $11 \pm 3 \mu\text{M}$.

These experiments are consistent with arecaidine and guvacine acting in rat brain slices as substrate-competitive inhibitors of GABA uptake, binding to the GABA carrier and penetrating the tissue. Such an action *in vivo* could contribute to the observed effects of arecaidine administered to mice, which includes reduction in spontaneous activity, exploration and motility². Antagonism of the inactivation of GABA might lead to prolongation of GABA-induced synaptic inhibition, such as that proposed for pentobarbitone, which at 5 mM inhibits (58%) the uptake and potentiates (46%) the release of GABA in rat brain slices¹⁴. Arecaidine, injected subcutaneously into mice in doses of $50\text{--}100 \text{ mg kg}^{-1}$ ($0.3\text{--}0.6 \text{ mM}$ if evenly distributed in body fluids), has been shown to prolong barbiturate narcosis and to inhibit spontaneous activity². We have shown that guvacine, injected intraperitoneally into mice in doses of $50\text{--}100 \text{ mg kg}^{-1}$ provoked a pronounced but rather short lived reduction of spontaneous activity, the duration of action being 15–30 min.

Guvacine is more potent than arecaidine with respect to inhibition of GABA uptake *in vitro*. The above mentioned results may stimulate further studies on the pharmacology of guvacine and arecaidine, and preparation of structural analogues of these amino acids as potential psychoactive agents.

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Persistence of tetracycline resistance in pig *E. coli*

THE practice of feeding tetracyclines to pigs to promote growth was prohibited in Britain in March 1971, after a period of some 17 yr during which this practice had been common. It was prohibited because it had encouraged the emergence of antibiotic-resistant strains of bacteria in pigs. A comparison of the results of examining pigs brought to Chelmsford Market in 1956, 1970 and July 1972 indicated that during 16 months prohibition the incidence of pigs harbouring tetracycline-resistant *Escherichia coli* in their faeces had not decreased but that the amount of these organisms in the pig population might have done so¹. To obtain additional information, similar surveys were done in July 1973, July 1974 and July 1975; the result of all six surveys are collated in this paper.

On each occasion, individual faecal specimens from 100 pigs in different pens were collected; this ensured that a high proportion of the pigs had come from different farms. The methods of assessing the antibiotic sensitivity of *E. coli* in the specimens, of recording the results and of identifying transmissible resistance in *E. coli* strains have been described previously¹. So has the method of identifying strains harbouring non-transmissible antibiotic resistance determinants that can be mobilised by transfer factors F, I or A2 (refs 2 and 3).

The results of the sensitivity tests, summarised in Table 1, suggest that in the four years since prohibition the amount of tetracycline-resistant *E. coli* in the pig population might have decreased slightly but that the incidence of pigs excreting these organisms (100% in 1975) had not. The failure of prohibition to achieve a greater impact may be because during the long period in which the *E. coli* in the pig population had been exposed to tetracycline, resistant strains had emerged which were now able to compete on equal terms with sensitive strains. The decrease since 1970 in the proportion of tetracycline-resistant strains whose resistance was transmissible (Table 2) suggests that, in a comparatively tetracycline-free environment, possession of a mechanism for transmitting tetracycline resistance is, in general, disadvantageous. The tetracycline-resistance determinants in 9 of 15 non-transmitting tetracycline-resistant *E. coli* strains isolated from different pigs in the 1973 survey could be mobilised by transfer factors F, I or A2. The continued veterinary use of tetracycline could also have contributed to the failure. The veterinary use of streptomycin and sulphonamides was undoubtedly responsible for a substantial amount of the streptomycin and sulphonamide resistance arising during the study period because these two antibiotics have not been used as feed additives. The use of either antibiotic could have contributed to this situation because of 144 streptomycin-resistant strains isolated from different pigs in the 1972–75 surveys, 127 were also sulphonamide resistant, probably because streptomycin and sulphonamide-resistance determinants are commonly linked on the same plasmid. The use of these antibiotics could also have made some contribution to the survival of tetracycline-resistant *E. coli* because 63 of the 144 strains previously mentioned were tetracycline resistant and 28 of 146 strains selected because they were tetracycline resistant were streptomycin resistant. The use of streptomycin was probably

Table 1 Antibiotic sensitivity of *E. coli* in faecal specimens of pigs

Antibiotic	Year when specimens were collected	% of specimens in which the <i>E. coli</i> were:			
		All resistant	Resistant and sensitive	Nearly all sensitive	All sensitive
Oxytetracycline	1956	18	13	22	47
	1970	64	22	7	7
	1972	36	41	16	7
	1973	23	62	13	2
	1974	25	53	18	4
	1975	41	52	7	0
Streptomycin	1956	0	0	2	98
	1970	15	45	20	20
	1972	7	42	30	21
	1973	10	58	22	10
	1974	8	54	24	14
	1975	13	55	25	7
Sulphonamides	1956	2	2	4	92
	1970	12	32	18	38
	1972	5	41	29	25
	1973	10	58	22	10
	1974	13	48	21	18
	1975	14	51	26	9
Chloramphenicol	1956	0	0	0	100
	1970	0	3	2	95
	1972	0	1	3	96
	1973	0	8	19	73
	1974	0	4	30	66
	1975	0	0	8	91
Ampicillin	1970	1	20	15	64
	1972	0	12	18	70
	1973	0	24	35	31
	1974	0	20	48	32
	1975	2	16	51	31
Neomycin	1970	1	1	5	93
	1972	0	1	1	98
	1973	0	5	7	88
	1974	1	0	7	92
	1975	1	2	5	92
Furazolidone	1970	1	3	4	92
	1972	1	1	6	92
	1973	0	4	13	83
	1974	0	0	2	98
	1975	0	0	1	98
Spectinomycin	1972	3	20	26	51
	1973	1	23	34	43
	1974	1	16	31	52
	1975	0	4	43	53
Polymixin, trimethoprim and sodium nalidixate	1972	0	0	0	100
	1973				
	1974				
	1975				

The method of classifying the specimens according to their content of resistant and sensitive *E. coli* has been described previously¹.

largely responsible for the finding of *E. coli* organisms resistant to the comparatively rarely used spectinomycin, all of 27 spectinomycin-resistant strains isolated from different pigs also being streptomycin resistant, a probable consequence of the fact that one form of plasmid-determined streptomycin resistance is mediated through an adenylating

enzyme that inactivates spectinomycin in addition to streptomycin. The increasing incidence of organisms possessing chloramphenicol resistance, most of which was transmissible, during 1970–74 gave some cause for concern. The concentration of these organisms in most of the faecal specimens, however, was very low and, fortunately, the increase was not maintained in the 1975 survey. That no *E. coli* organisms resistant to trimethoprim, polymixin or nalidixic acid were found in any of the surveys was probably a reflection of the very low degree of exposure of the pig population to these antibiotics.

The failure of prohibition of the growth promotion use of tetracyclines to markedly reduce the amount of tetracycline-resistant *E. coli* in the pig population stresses the fallacy of assuming that the ecological changes brought about largely by the persistent and widespread use of antibiotics can be reversed simply by resorting to a policy of withdrawal. It serves also to emphasise the need of strict adherence to the main recommendation of the Swann Report⁴ that antibiotics commonly used in human and veterinary medicine should

Table 2 Incidence of transmissible resistance in *E. coli* strains isolated at different times

Resistance studied	Year of isolation	No. of resistant strains examined	% Whose resistance was transmissible
Tetracycline	1970	30	73
	1972	30	60
	1974	50	32
	1975	50	36
Streptomycin	1970		
	1972	28	46
	1974	50	28
	1975	50	40

not be used as feed additives for growth promotion so that such undesirable occurrences can be avoided in the future.

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Genetic transfer of nitrogenase–hydrogenase activity in *Rhodospseudomonas capsulata*

THE transfer of nitrogen fixation (*nif*) genes to bacterial mutants defective in this capacity (*nif*[−]) by transduction¹ and conjugation² was first observed in 1971 with *Klebsiella pneumoniae*. The investigations cited and the subsequent demonstration³ that *nif* genes can be transferred from *K. pneumoniae* to *Escherichia coli* have been widely regarded as harbingers of the molecular biology of the nitrogen fixation process. We now report a new system of interest in this connection, namely, the transfer of *nif* genes in the non-sulphur purple photosynthetic bacterium *Rhodospseudomonas capsulata*. Genetic transfer in this organism is mediated by a filterable vector of small size and unknown nature, the “gene transfer agent” (GTA), discovered by Marrs⁴.

Mutants of *Rps. capsulata* unable to fix N₂ were isolated from wild type strain B10 (obtained from Dr B. Marrs; see ref. 4) as follows. Cells were grown photosynthetically (anaerobically) at about 30 °C in a synthetic medium containing DL-malate and 0.1% ammonium sulphate as the carbon and nitrogen sources, respectively (medium RCVBN described in ref. 5). A culture in the late exponential growth phase was treated with N-methyl-N′-nitro-N-nitrosoguanidine (100 µg ml^{−1} for 30 min) and the mutagen was then removed by washing the cells several times by centrifugation. The cells were resuspended in the synthetic medium, incubated for about 18 h anaerobically in darkness, and then illuminated to permit growth and segregation of mutant phenotypes. After the early stationary phase was reached, cells were transferred into synthetic medium from which (NH₄)₂SO₄ was omitted, and the anaerobic culture incubated under illumination until formation of gas bubbles was evident (H₂ formation occurs only after all exogenous ammonium sulphate has been used). To enrich for *nif*[−] mutants, penicillin G was added (10 U ml^{−1}) and the culture was illuminated anaerobically in a GasPak jar (atmosphere: N₂ + H₂ + CO₂) for 14 h; excess penicillinase was then added and the suspension was left to stand for 1 h at room temperature. Mutants with the *nif*[−] phenotype were sought by plating samples on synthetic medium agar containing a decreased concentration of (NH₄)₂SO₄ (0.01%); the plates were incubated anaerobically (GasPak jars) in the light. In these conditions, the final diameter of putative *nif*[−] colonies is considerably smaller than that of *nif*⁺ colonies (able to use N₂) and the former can consequently be easily identified. Several isolates obtained in this way were unable to use N₂ as a nitrogen source for growth and we consider here the properties of five of them (Table 1). Appropriate tests showed that the mutants retained identifiable characteristics of the parental strain, such as photosynthetic growth capacity and persistence of a specific bacteriophage associated only with this strain⁶.

Tests for the presence of nitrogenase in mutants W12, W15 and W16 by the sensitive acetylene reduction assay⁷ gave negative results (using cells starved for nitrogen or grown with nitrogen sources that do not repress nitrogenase synthesis). These strains cannot use N₂ for growth, but in common with

Table 1 Conversion of *nif*[−] *Rps. capsulata* mutants to *nif*⁺ phenotype by GTA from wild type B10 cells

<i>Nif</i> [−] mutant	<i>Nif</i> ⁺ colonies per plate	
	Control	+GTA
W11	3	917
W12	1	298
W13	1	1,320
W15	4	1,012
W16	6	450

GTA activity was assayed as described by Wall *et al.*⁸, essentially using procedures developed by Marrs⁴ and Solioz *et al.*¹³. GTA filtrates (0.3 µm Millipore filters) were obtained from early stationary phase cultures of *Rps. capsulata* strain B10 grown photosynthetically in a medium consisting of 0.3% Difco yeast extract, 0.3% Difco Bactopeptone, 2 mM CaCl₂ and 2 mM MgSO₄ (YPS medium); appropriate controls showed that the filtrates did not contain viable cells. The mutants were also grown photosynthetically in YPS medium; cells were collected in the early stationary phase and resuspended in the original volume of buffer composed of 10 mM Tris-HCl (pH 7.8), 1 mM CaCl₂, 1 mM MgCl₂, 1 mM NaCl and bovine serum albumin (500 µg ml^{−1}). To test the effect of GTA, equal volumes of filtrate and recipient cell suspension were mixed and the resulting suspension was incubated semi-aerobically in darkness for 1 h at 34 °C. Aliquots of 0.2 ml were then plated as an overlay (in the buffer noted + 0.3% Wilson Ionagar) on an underlay of RCVBN synthetic medium, modified by omission of ammonium sulphate, solidified with 1.2% Ionagar. The plates were placed in anaerobic jars containing N₂ (GasPak system) which were allowed to stand at room temperature for 2 h before incubation under illumination at 30–33 °C. Control values are numbers of spontaneous *nif*⁺ revertants observed per 3–4 × 10⁸ cells plated. Values under +GTA are numbers of *nif*⁺ colonies observed per 0.1 ml of B10 filtrate used (or per 3–4 × 10⁸ recipient cells plated after incubation with B10 filtrate). The numbers of transferants observed in such experiments vary considerably from day to day, apparently because of slight differences in conditions affecting the physiological states of donor and recipient cells^{6,13}.

the B10 parental strain can utilise ammonium sulphate, urea, glutamate, glutamine, aspartate, proline, ornithine and thymine as nitrogen sources. Although mutants W11 and W13 show the *nif*[−] phenotype, nutritional and genetic experiments indicated that the lesions in these strains are different, and probably associated with defects in synthesis or metabolism of glutamine or glutamate. The data in Table 1 show that GTA produced by cultures of the *nif*⁺ B10 (parental) strain can be accepted by the *nif*[−] mutant strains, resulting in ‘transferants’ which have regained the capacity to fix N₂. The second column (control) shows the frequency of spontaneously occurring *nif*⁺ revertants in the mutant populations; the last column shows the greatly increased number of *nif*⁺ colonies seen after the mutants are treated with GTA obtained from B10 cultures.

The possibility that strains W12, W15 and W16 have the same *nif*[−] mutation was tested by examining the capacities of filtrates from cultures of each mutant to restore the *nif*⁺ character to cells of the other strains. Table 2 shows that filtrate from a particular mutant does not have activity with cells of the same strain. GTA from strain W12 gave *nif*⁺ recombinants with cells of W15 and W16, and similarly, GTA from W15 restored the *nif*⁺ trait in strains W12 and W16. We conclude that the mutations in the three strains are not identical. Although mutant W16 accepts GTA from strains W12 and W15, it is inactive as a GTA donor. The apparent inability of W16 to

Table 2 Recombination between *nif*[−] mutants

Culture filtrate	Recipient cells		
	W12	W15	W16
W12	—	+	+
W15	+	—	+
W16	—	—	—

Procedures for growth of cells, preparation of filtrates and GTA tests were as described for Table 1. +, *nif*⁺ recombinants observed in numbers significantly greater (> tenfold) than the spontaneous reversion rate. —, Number of *nif*⁺ colonies approximately the same as observed with recipient cells not treated with culture filtrate (that is, spontaneous reversion rate).

produce GTA was checked using another assay, namely, capacity to produce GTA required for restoration of photosynthetic growth competence to a non-photosynthetic white mutant of *Rps. capsulata*⁶. GTA preparations from W12 and W15 showed activity in the latter test, but filtrates from W16 did not. The peculiarities of W16 in this respect are still unexplained and are under investigation.

Typical purple bacteria produce large quantities of molecular hydrogen during photoheterotrophic growth on organic acids (for example, malate) when certain amino acids are used as nitrogen sources (for example, glutamate)⁸. Addition of ammonia, the first stable product of N_2 fixation, causes repression of synthesis of the H_2 -evolving system of *Rhodospirillum rubrum*⁹, and this is also observed with *Rps. capsulata*. In contrast to the parental B10 strain used in the present studies, *Rps. capsulata* mutants W12, W15 and W16 are unable to produce H_2 during photosynthetic growth in malate+glutamate medium (on occasion, a small quantity of H_2 is observed with mutant W12, which is also leaky with regard to fixation of N_2). All the *nif*⁺ spontaneous revertants and transferants of these mutants tested so far, however, showed active production of H_2 . These observations strongly support the suggestion¹⁰ that in photosynthetic bacteria, nitrogenase and H_2 -evolving hydrogenase activities are catalysed by the same enzyme complex. Although other explanations are conceivable (for example, that the two activities in question are attributable to different enzyme systems that are coordinately regulated) we consider that the data available are most plausibly interpreted on the basis of dual functions of the nitrogenase complex.

Studies with cell-free preparations from various N_2 -fixing bacteria have revealed that nitrogenases can evolve H_2 by an energy-dependent mechanism^{11,12}; photoproduction of H_2 by non-sulphur purple bacteria appears to be the most clear-cut example where such H_2 formation occurs as a normal and major physiological process. The latter is believed to represent a regulatory device that enables the cell to dispose of reducing power and adenosine triphosphate when these are generated in excess of biosynthetic demands⁸; that is, a device that facilitates energy idling. It is worth noting that use of H_2 as an electron donor for photoautotrophic growth apparently is mediated by a different hydrogenase system. This is indicated by the fact that *Rps. capsulata* mutants W12, W15 and W16 grow readily, photosynthetically, with 0.1% $(NH_4)_2SO_4$ as the nitrogen source and H_2+CO_2 as the sole sources of reducing power and carbon, respectively.

Soloz et al.¹³ found that GTA can be used to demonstrate linkage between genes for carotenoid biosynthesis and those involved in production of bacteriochlorophyll in *Rps. capsulata*. This development and the findings reported here indicate that GTA can be exploited for more refined analysis of the molecular biology of the nitrogenase-hydrogenase system.

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Characterisation of a rotavirus

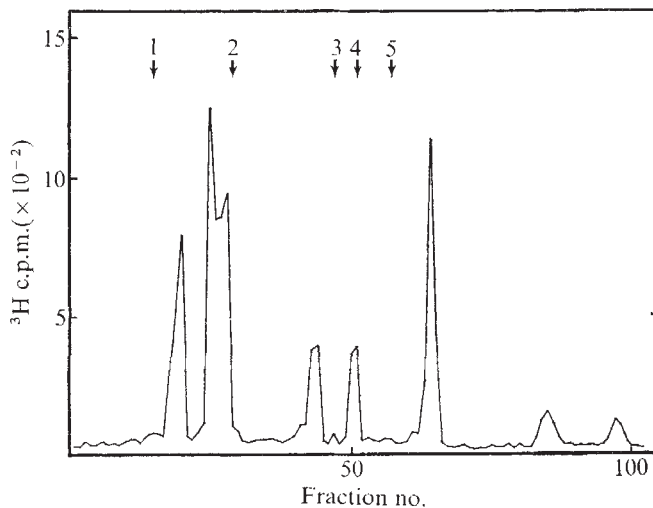
ACUTE gastroenteritis is one of the most common causes of illness in children, calves and piglets, and causes severe economic loss in domestic animals. Although some outbreaks of gastroenteritis are associated with bacterial pathogens, particularly *Escherichia coli*, no known pathogens had been isolated from a significant proportion of outbreaks until recently, when viruses with a distinctive morphology and antigenic similarity were found associated with diarrhoea in piglets, calves and children^{1,2}. These viruses are major pathogens and it has also been shown that viruses isolated from man are infectious for the pig³. Initially, these viruses were described as reovirus-like⁴ but subsequently the names rotavirus⁵ and duovirus⁶ were proposed. They are morphologically and antigenically distinct from both the reoviruses and orbiviruses^{5,7} and also differ from them in some of their physicochemical properties⁸.

Studies of the structure of rotaviruses have been confined to the nucleic acid of the calf virus and the related virus of epizootic diarrhoea of infant mice (EDIM). Both viruses were shown to contain RNA^{9,10}, and Welch and Thompson⁸ suggested that the calf virus RNA might be double stranded. The results of the experiments described here show that calf rotavirus possesses several of the features characteristic of the reo- and blue tongue viruses and should be classified with them¹¹.

To facilitate purification of the virus and to confirm the type of nucleic acid, secondary calf kidney cell cultures in 40-ounce roller bottles were infected with a strain of tissue culture adapted calf rotavirus¹² and maintained with Earle's medium containing 2% foetal calf serum and either ³H-uridine or ³H-thymidine. A total of 100 μ Ci isotope was added to each bottle over a period of 3 d. The cells and maintenance medium were frozen and thawed three times, the cell debris removed by centrifuging at 2,000g for 5 min and the virus purified by the following method.

The virus was pelleted by centrifuging at 40,000g for 1 h, resuspended in 0.1 M Tris, pH 7.6, and the insoluble debris

Fig. 1 Separation of the different segments of the ³H-uridine-labelled RNAs of calf rotavirus by polyacrylamide gel electrophoresis in 5% gels for 24 h at 3.5 mA per gel. The molecular weights were estimated by reference to the migration of ¹⁴C-adenine-labelled reovirus RNAs in the same gel. Arrows indicate the positions of the reovirus RNAs: (1) $2.6\text{--}2.8 \times 10^6$; (2) $1.5\text{--}1.6 \times 10^6$; (3) 0.9×10^6 ; (4) 0.8×10^6 ; (5) 0.6×10^6 (ref. 14).



removed at 2,000g. The supernatant fluid, which contained a large number of rotavirus particles, was mixed with 0.5% sodium deoxycholate and centrifuged at 40,000g for 1 h in a 20-ml sucrose gradient (15–45% in 0.1 M Tris, 0.1 M NaCl, pH 7.6) layered over a 1-ml cushion of saturated CsCl solution. Fractions (1 ml) were collected and assayed for radioactivity. The cultures which contained ^3H -thymidine gave a band of radioactivity at the caesium chloride-sucrose interface with the remainder of the radioactivity at the top of the gradient. The ^3H -uridine-labelled cultures also gave a band of radioactivity at the CsCl-sucrose interface, but in addition to the radioactivity at the top of the gradient, there was also a peak at about 500S. This peak contained the major part of the infectivity, and electron microscopical examination showed the presence of a large number of rotavirus particles confirming Welch's observation that the virus contained RNA⁹. Very few particles were found elsewhere in the gradient.

The relative sedimentation coefficient of the virus was determined by cosedimenting a mixture of the ^3H -labelled virus and unlabelled reovirus in a sucrose gradient. Well separated peaks of radioactivity and ultraviolet absorbing material were obtained. Using a value of 630S for reovirus, a relative value of 500S was calculated for the rotavirus.

The buoyant density of the virus was determined by centrifuging a mixture of ^3H -rotavirus and ^{32}P -Maus-Elberfeld virus at 60,000g for 17 h in 5 ml preformed CsCl gradients (density range 1.30–1.40 g ml⁻¹). The distribution of radioactivity showed that the two viruses sedimented as sharp peaks with densities of 1.36 and 1.33 g ml⁻¹ respectively. The value for the Maus Elberfeld virus is in close agreement with that found by Rueckert and Schäfer¹³, and the density determined for the rotavirus agrees with the value found by Welch⁹. When the rotavirus suspension was centrifuged in CsCl without first treating with deoxycholate, two distinct bands at densities of 1.36 and 1.38 g ml⁻¹ were obtained which contained rotavirus particles with and without their outer capsid layer, respectively (J.C.B. and G.N.W., unpublished). A similar result was obtained when the peak of ^3H -uridine radioactivity located at the CsCl-sucrose interface in the purification procedure described here was centrifuged in a CsCl gradient.

The RNA was extracted from the purified ^3H -rotavirus by diluting the fractions from the 15–45% sucrose gradient with 5 volumes 0.1 M Tris, pH 9.0, containing 0.5% SDS, and shaking with phenol. All the radioactivity was found in the aqueous phase, which was mixed with ribosomal RNA prepared from BHK21 cells and the mixture of RNAs precipitated with 2 volumes ethanol at -20 °C. To determine whether rotavirus RNA was single or double stranded,

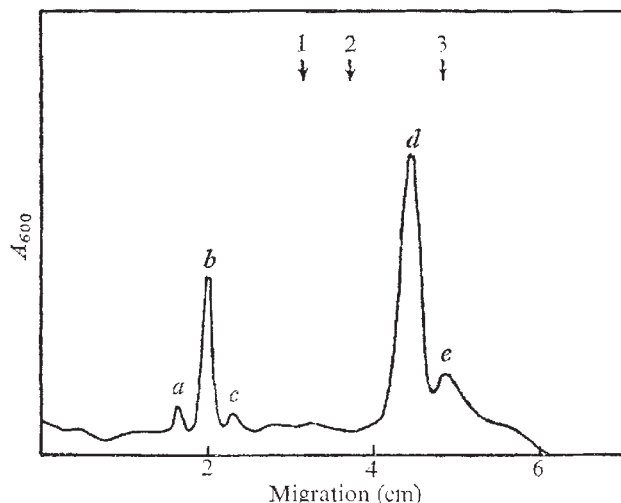


Fig. 2 Separation of the polypeptides of calf rotavirus by polyacrylamide gel electrophoresis in 7.5% gels for 16 h at 5mA per gel. The molecular weights were estimated by reference to the migration of ^{35}S -methionine-labelled VSV proteins in the same gel. Arrows indicate the positions of the marker proteins: (1) 69×10^3 ; (2) 50×10^3 ; (3) 29×10^3 (ref. 15).

the precipitate was dissolved in 2 ml 0.04 M phosphate, pH 7.6 and 1 ml was incubated with 0.01 μg pancreatic RNase at 20 °C for 15 min. The untreated and RNase-treated samples were then centrifuged at 35,000g for 18 h in separate 5–25% sucrose gradients in 0.1% SDS-0.1 M acetate, pH 5.0. The distribution of radioactivity was the same in both gradients with a broad peak at 11–16S but the A_{260} profiles showed that the single-stranded ribosomal RNA had been hydrolysed in the sample treated with RNase. The broad peak of radioactivity suggested that the virus RNA was heterogeneous and this was confirmed by electrophoresis in 5% polyacrylamide gels for 24 h at 3.5 mA per gel, when eight peaks of radioactivity were obtained (Fig. 1). To enable their molecular weights to be calculated, a mixture of the RNAs of ^3H -uridine rotavirus RNA and ^{14}C -adenine reovirus, type 3, was electrophoresed in the same conditions and 1-mm gel slices counted for each isotope. Using the molecular weights given by Martin and Zweerink¹⁴ for the reovirus RNAs, values of 2.2, 1.9, 1.7, 0.9, 0.8, 0.5, 0.3 and 0.2×10^6 were calculated for the rotavirus RNAs. The relative radioactivity of each peak indicated that the two peaks with molecular weights of 1.9 and 0.5×10^6 consisted of two and three or four species respectively. This means that the rotavirus RNA consists of 11 or 12 segments with a total molecular weight of 11×10^6 – 12×10^6 .

Table 1 Comparison of the properties of reovirus, blue tongue virus and calf rotavirus

Property	Reovirus		Blue tongue virus		Calf rotavirus (present work)	
Sedimentation coefficient	630		550		about 500	
Diameter (nm)	60–75		69		60–65	
Buoyant density in CsCl (g ml ⁻¹)	1.38		1.36		1.36–1.38	
RNA and proteins	Polypeptide	RNA	Polypeptide	RNA	Polypeptide	RNA
	MW $\times 10^{-3}$	MW $\times 10^{-6}$	MW $\times 10^{-3}$	MW $\times 10^{-6}$	MW $\times 10^{-3}$	MW $\times 10^{-6}$
	155	2.5 (2.8)	155	2.7 (2.8)	135	2.2 (2.4)
	140	2.4 (2.5)	110	2.0 (2.0)	122	1.9 (2.2)
	—	2.3 —	100	1.9 (1.8)	96	1.7 (1.7)
	80 and 72	1.6 (1.4)	72	1.3 (1.3)	—	0.9 —
	42	0.9 (0.8)	58	1.1 (1.0)	36	0.8 (0.7)
	38	0.8 (0.7)	38	0.6 (0.7)	30	0.5 (0.5)
	34	0.6 (0.6)	32	0.6 (0.6)	—	0.3 —
	—	—	—	0.5 —	—	0.2 —
Capsid proteins (species)	7		7		5	
Total number of double-stranded	10		10		11–12	
RNA segments	10		10		11–12	
Total molecular weight of RNA	14.7×10^6		12×10^6		11 – 12×10^6	

Figures in parentheses indicate the double-stranded RNA molecular weight (MW) required to code for each polypeptide, assuming a polypeptide to double-stranded RNA ratio of 18 (ref. 14).

Data on reo- and blue tongue viruses were obtained from refs 11 and 14, respectively.

The polypeptides of the virus were examined by polyacrylamide gel electrophoresis. Purified virus was digested at 37 °C for 1 h with 8 M urea, 1.0% SDS, 0.1% mercaptoethanol, dialysed against 0.5 M urea, 0.1% SDS, 0.1% mercaptoethanol in 0.01 M phosphate, pH 7.2, and the mixture of polypeptides electrophoresed in 7.5% polyacrylamide gels for 16 h at 5 mA per gel. Staining with 0.5% Coomassie blue revealed the presence of at least two major bands (*b* and *d*) and three minor bands (*a*, *c* and *e*, Fig. 2). The two minor bands *c* and *e* were present in variable amounts in different preparations. For an assessment of their molecular weights, a mixture of the rotavirus polypeptides and a quantity of ^{35}S -methionine-labelled vesicular stomatitis virus polypeptides which was insufficient to stain with Coomassie blue was coelectrophoresed in the conditions described above. The gel was stained to ascertain the positions of the rotavirus polypeptides and then cut into 1-mm slices for counting (Fig. 2). The molecular weights of the rotavirus polypeptides were calculated to be 135, 122, 96, 36 and 30×10^3 . A possible coding relationship between these polypeptides and the RNAs is shown in Table 1.

Although the calf rotavirus has a smaller sedimentation coefficient and is morphologically distinct from the reo- and blue tongue viruses, the segmented and double-stranded nature of its RNA and the number and molecular weights of the RNA and polypeptides suggest that it should be regarded as a member of the reo- and blue tongue virus group. Its precise relationship to the other members of the group, however, will require extensive cross hybridisation studies of the virus RNAs.

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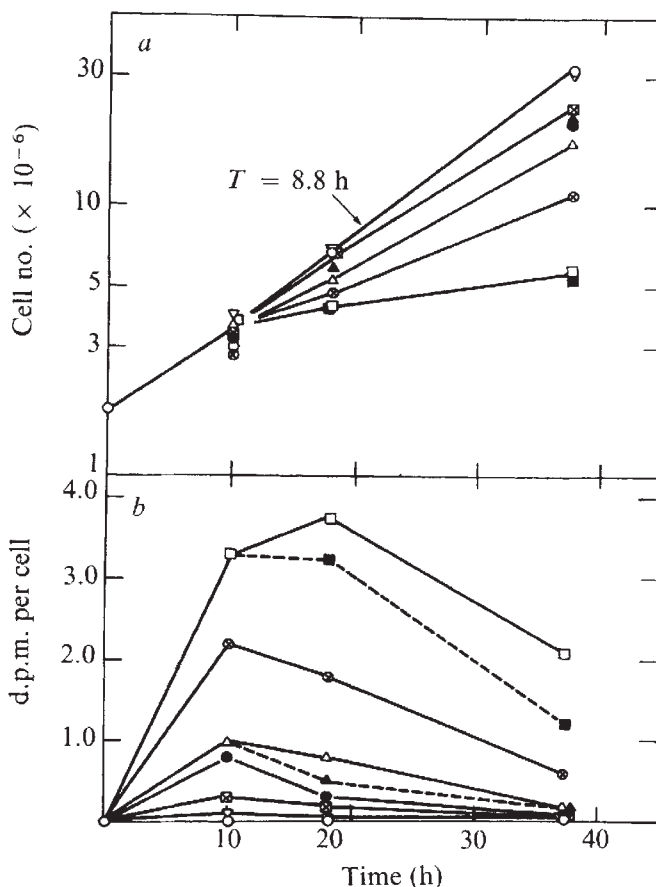
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biological anomalies, including mutations, chromosome aberrations, cell death³, growth retardation and strand breaks in the DNA molecule⁴ (for reviews see refs 5 and 6). At least one investigator⁵ has advocated separate evaluation of the potential perturbations in every experimental system using radioisotope-labelled nucleic acid precursors.

Because the first step in many experiments is lengthy labelling (for example, overnight) with a radioactive DNA precursor, and because our unpublished observations indicate unusual growth behaviour by several mammalian cell lines in the presence of tritiated thymidine (^3H -TdR), we investigated the influence of this precursor on the cycle progressions of those mammalian cell lines by high speed flow microfluorometry (FMF)^{7,8}. With this technique, the cells in the G_1 , S , and $G_2 + M$ phases of the cell cycle can be counted by direct measurement of the DNA content per cell. Representative data obtained with one cell line are presented in preliminary form now because they exemplify growth perturbations that must be considered when studies with radioactive precursors are interpreted.

Monolayer cultures of Chinese hamster fibroblasts (V79-182) were cultured as described before^{9,10}. Petri dishes (100 mm, Falcon) were inoculated with equal numbers of V79-182 cells in FST-15 medium (Eagle's basal medium supplemented with antibiotics, 4% NCTC-109, trypsin, glutamine and 15% undialysed foetal calf serum). After overnight incubation at 37 °C to provide exponential populations (doubling time about 9 h), FST-15 was replaced with fresh, warm (37 °C) EM-15 medium (FST-15 without NCTC-109 or trypsin)

Fig. 1 Cell proliferation (*a*) and ^3H d.p.m. per cell (*b*) of V79 fibroblasts after continuous incubation in the presence of various concentrations of ^3H -TdR. Fresh growth medium containing ^3H -TdR was added to exponentially growing cultures at zero time: $\circ$, control (no ^3H -TdR); ∇ , 0.025 $\mu\text{Ci ml}^{-1}$; crossed square, 0.075 $\mu\text{Ci ml}^{-1}$; $\triangle$, 0.25 $\mu\text{Ci ml}^{-1}$; $\blacktriangle$, 0.25 $\mu\text{Ci ml}^{-1}$, removed after 10 h; crossed circle, 0.5 $\mu\text{Ci ml}^{-1}$; $\square$, 1.0 $\mu\text{Ci ml}^{-1}$; $\blacksquare$, 1.0 $\mu\text{Ci ml}^{-1}$, removed after 10 h; $\bullet$, 20 $\mu\text{Ci ml}^{-1}$, flash exposure (20 min). *T*, mean doubling time of control populations.



Perturbations in cell cycle progression from radioactive DNA precursors

RADIOACTIVE precursors of DNA have widespread application in eukaryotic cell systems for studies of kinetic phenomena, such as DNA metabolism and cell population growth, and for labelling DNA^{1,2} for biophysical or biochemical investigations. Implicit in their use is the assumption that the experimental endpoints are not perturbed by the presence of the labelling isotope(s). But the validity of this assumption requires re-evaluation in the light of reports that the incorporation of radioactive compounds into cellular DNA causes significant

containing ^3H -methyl-thymidine (6.7 Ci mmol^{-1} , New England Nuclear Corp.) at concentrations varying from 0.025 to $1.0 \mu\text{Ci ml}^{-1}$ for continuous labelling or at $20 \mu\text{Ci ml}^{-1}$ (15 Ci mmol^{-1} , New England Nuclear Corp.) for flash labelling. After various periods of incubation at 37°C , the radioactive medium was aspirated from a group of dishes and the cells were washed twice with 10 ml of FST-15 medium supplemented with non-radioactive ($5 \mu\text{g ml}^{-1}$) thymidine and then trypsinised into suspension. Each sample was divided into three aliquots: the first was assayed for cell number with a haemocytometer; the DNA radioactivity in the second aliquot was determined by liquid scintillation spectrometry after precipitation of the DNA with perchloric acid; the third aliquot was prepared for DNA analysis by FMF according to the procedures of Van Dilla *et al.*^{7,8,11,12}. These procedures determine the

distribution of cells throughout the cell cycle by measuring the DNA content of each individual cell and then constructing a spectrum of cellular DNA contents for the entire population^{7,8,13}. The DNA within the cell is stained with a fluorescent dye such as acriflavine, and the cell population is passed through a laser beam that excites the dye. The resulting fluorescence from each cell is proportional to the amount of fluorescent dye bound to the DNA within the cell, and thus is proportional to the DNA content of that cell^{7,8}. At least $50,000$ cells were assayed for every DNA spectrum given in the figures. Some FMF measurements were made with the fluorometer at the Los Alamos Laboratory of the University of California^{7,8}; the others were made with a similar instrument at the Lawrence Livermore Laboratory of that university.

Incorporation of ^3H -TdR affected the growth of cells

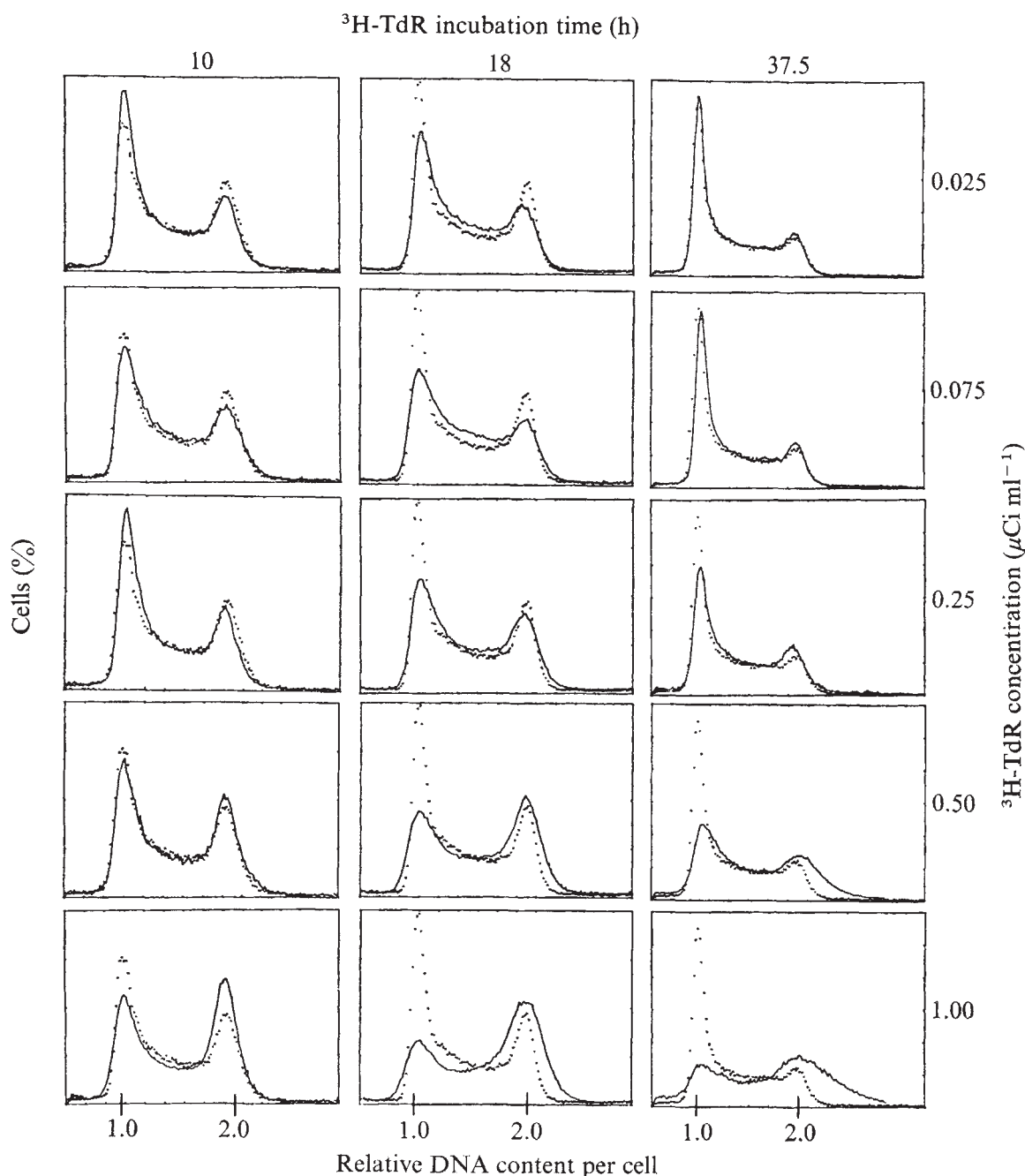


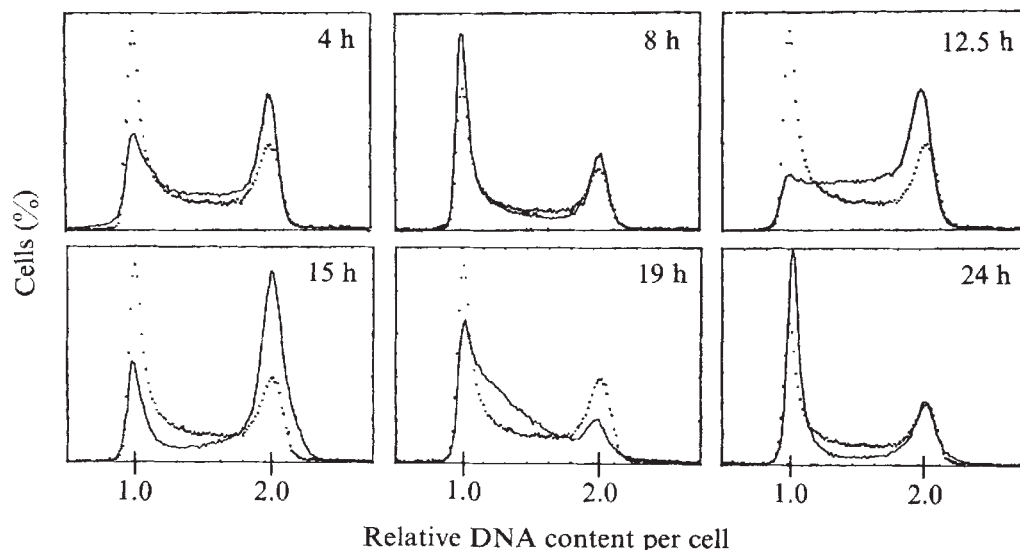
Fig. 2 Distribution of DNA contents of V79 fibroblasts as a function of incubation time with graded concentrations of ^3H -TdR. ^3H -TdR, at the desired concentration in fresh growth medium, was added to the exponentially growing cultures at zero time. Samples of ^3H -TdR-treated cells (—) and control cells (....) were prepared for FMF after various incubation times. The positions of the G_1 and the $G_2 + M$ peaks are represented by 1.0 and 2.0 relative DNA contents, respectively, and the area between the peaks represents the S-phase cells.

(Fig. 1a) even when the precursor was depleted substantially after one cell generation (Fig. 1b). The cell counts taken after 10 h of incubation were statistically the same for all samples by the *F* test. But after 18 and 37.5 h (equivalent to more than four doubling times for controls), the growth rates decreased progressively once the concentration of the precursor exceeded $0.025 \mu\text{Ci ml}^{-1}$. To verify that the precursor was depleted from the medium during the first doubling time, the medium was removed from two of the samples after 10 h and replaced with fresh non-radioactive medium. As expected, this treatment lowered the radioactivity in the cells only slightly and the subsequent growth rates were virtually unchanged (Fig. 1).

is a common experimental technique, we also did a 20-min flash-labelling experiment using ^3H -TdR at $20 \mu\text{Ci ml}^{-1}$. Four hours after flash labelling, a large fraction of the cell population had a $G_2 + \text{M}$ DNA content (Fig. 3). After another 4 h, the cells had overcome the block and progressed into G_1 (Fig. 3) in a semi-synchronous fashion. This synchrony evidently persisted for at least two cell generations (Fig. 3). Exposure to non-radioactive thymidine at the same concentrations as used for ^3H -labelled cells ($1.33 \mu\text{M}$), either pulsed or continuously, did not change the DNA distributions from those found with control populations (data not shown).

The perturbations in growth of V79 cells caused by ^3H -TdR

Fig. 3 Distribution of DNA contents of V79 fibroblasts as a function of time after exposure to a flash label (20 min) of ^3H -TdR ($20 \mu\text{Ci ml}^{-1}$, 15 Ci mmol^{-1}). After the 20-min incubation with ^3H -TdR, the medium was aspirated and replaced, after several rinses, with fresh, warm growth medium. ^3H -TdR-treated cultures (—) were fixed after various incubation times and compared to untreated controls (···). The positions of the G_1 and the $G_2 + \text{M}$ peaks are represented by 1.0 and 2.0 relative DNA contents, respectively. The area between the peaks represents the S phase cells.



The distribution of DNA contents per cell for control samples (Fig. 2, dotted lines) at 10 h and 18 h were those expected for rapidly dividing, exponential populations^{7,8,11-13}; the change at 37.5 h indicates a greater proportion of cells with a G_1 content as the confluent population approached the plateau phase of growth. But some of the DNA profiles for cells incubated in the presence of ^3H -TdR (Fig. 2, solid lines) exhibited abnormalities, the magnitude and time of appearance of which depended on the level of radioactivity. For cell populations incubated with high levels of ^3H -TdR (0.5 and $1.0 \mu\text{Ci ml}^{-1}$), the most striking abnormality was the presence of an unusually large $G_2 + \text{M}$ peak, indicating a cycle block. This was noticeable after 10 h and persisted until the last sampling time of 37.5 h. Although G_2 cells cannot be distinguished from M cells by fluorometry, we believe that most cells blocked in $G_2 + \text{M}$ were actually G_2 cells because microscopic examination did not reveal the presence of unusually high numbers of mitotic cells. The possibility that late-S cells contributed to the $G_2 + \text{M}$ peak cannot be ruled out. The shape of the $G_2 + \text{M}$ peak was also abnormal. At 37.5 h, for example, the peak for the $1.0 \mu\text{Ci ml}^{-1}$ sample had a long tail, which indicates the presence of cells with DNA complements greater than normal G_2 or M cells (4C DNA). Concomitantly, the G_1 peak in this sample contained cells with less DNA than G_1 controls. Other indications of the severity of the effects of the highest level of radioactivity were the presence of many giant cells and some necrotic cells in the $1.0 \mu\text{Ci ml}^{-1}$ samples at 18 h and 37.5 h.

With low ^3H -TdR concentrations (up to $0.075 \mu\text{Ci ml}^{-1}$), the block in the $G_2 + \text{M}$ phase of the cycle was transient. The cells had overcome this block by 10 h, and the resulting semi-synchrony produced populations with more cells in G_1 and early S than in control populations (Fig. 2). This cell cycle perturbation was even more evident one generation later, at 18 h, but by 37.5 h the DNA profiles were again like those of the control populations.

Because flash labelling with ^3H -TdR of high specific activity

in these experiments are unequivocal. Perturbations analogous to those demonstrated here, but with different severity, were observed for Chinese hamster ovary fibroblasts, L5178Y murine leukaemic lymphoblasts, Chang's liver cells (LICH) and human skin fibroblasts (WI-38). The most prominent effect on the DNA profiles for all these cell types was the appearance of a $G_2 + \text{M}$ block, the severity and length of which depended on the level of incorporated radioactivity. This expression of damage from internal irradiation by ^3H -TdR is similar to the G_2 block exhibited by cells that have been irradiated with external sources of ionising radiation^{11,12}. Strict comparison of the dose from an external source with the internal β particle of ^3H -TdR is not possible without further experimentation because of the localisation of the tritium decay within the cell nucleus and the chronic nature of the β irradiation, which is complicated by both the rate of uptake of ^3H -TdR and the rate of cell division. In these experiments, however, a rough estimate of the dose received during the first generation by cells grown in medium with ^3H -TdR at $0.5 \mu\text{Ci ml}^{-1}$ for that time is 200 rad (one ^3H -TdR disintegration ≈ 0.35 rad, refs 3 and 4).

Many investigators have emphasised the need for caution when radioactive DNA precursors are used in experiments because of their deleterious effects on cells (see review by Wimber⁵). Experimental results will be misleading if the endpoints measured are affected by incorporated radioactivity. For example, DNA repair mechanisms in externally irradiated eukaryotic cells should be studied with the lowest possible levels of incorporated radioactivity in order to permit precise evaluation of specific radiation responses^{14,15}. Our data emphasise the need for caution in the use of radioactive DNA precursors because perturbations in the cell cycle occur at or below the precursor levels in general use in cell biology. Simple cell counting may not be sufficient to determine alterations in cycle progression caused by labelling. For example, in our experiments, cell counts taken 10 h (that is, one cell doubling) after the addition of ^3H -TdR to the medium indicated that

growth had occurred normally, yet some of the DNA profiles already showed abnormal cell cycle distributions. Thus, it is clear that when radioactive DNA precursors are used as labels in experiments, accurate interpretation of the experiments will require determination of the exact locations of radioisotope-induced blocks in the cell cycle, the relative effects of DNA precursors labelled with different isotopes (for example, ^{14}C -TdR, ^{125}I -uridine), the relative effects of uniform and flash labelling, and the differences in sensitivities of different cell types.

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Replication of human KB cell DNA after infection by adenovirus type 12

LYTIC infection of human KB cells by adenovirus type 12 (ad 12) results in the early inhibition of host cell DNA synthesis¹. We have looked at some aspects of cell DNA replication after infection of KB cells by ad 12. We observed that the ligation of the Okazaki fragments is slower in cells affected by this virus. We also observed that the inhibition of cell DNA synthesis by this virus is not a random process and that certain classes of DNA, distinguished by their higher buoyant density and by their high reiteration frequency, are selectively synthesised.

Human KB cells were prelabelled with ^{14}C -thymidine (^{14}C -TdR) for 15-24 h and then infected with ad 12 or mock infected. After 8 h of infection cells were pulse labelled with ^3H -TdR for 15 min and then chased for the indicated times. The cells were then lysed and their DNA

was analysed by alkaline sucrose gradient centrifugation². As Fig. 1 shows, most label in a 15-min pulse is incorporated into lower molecular weight DNA in both infected and control cells. This is consistent with the Okazaki³ model of DNA replication observed also in other cell types⁴.

The small fragments were chased into higher molecular weight DNA. The process was essentially completed by 4 h in the mock infected cells. This was shown by the cosedimentation of pre-existing ^{14}C -DNA with the newly synthesised ^3H -DNA. Chain elongation was slower in the infected cells (Fig. 1).

In a randomly growing culture of cells the buoyant density of the newly synthesised DNA is the same as that of pre-existing DNA (Fig. 2a). Cells were prelabelled with ^{14}C -TdR and infected or mock infected. After 8 h of infection, they were pulse labelled with ^3H -TdR for 1 h with or without chasing for up to 6 h (no difference was observed with or without chasing). The cells were lysed and treated with Pronase and then centrifuged to equilibrium in neutral CsCl gradients. As Fig. 2b shows, the density of the newly synthesised DNA is higher than that of pre-existing DNA in the infected cells. The higher buoyant density is not due to single strandedness of the newly synthesised DNA or to the presence of single-strand tails in the infected cell DNA since the shift is still observed when DNA is treated with S_1 nuclease^{7,8} before its centrifugation in CsCl gradients (Fig. 2 inset). The observed shift in density is more pronounced if deoxycytidine is used as label (Fig. 2d). This is expected if the change in density is due to higher GC content of the newly synthesised DNA in the infected cells.

Table 1 Hybridisation of newly synthesised KB cell DNA to immobilised viral DNA

Sample	Input (c.p.m.)	Bound (c.p.m.)	% Bound
Empty filters	5,305	59	1.1
Viral DNA	5,305	3,240	61.1
Control (heavy side)	85,638	525	0.61
Control (light side)	50,128	301	0.6
Infected (9 h heavy side)	13,217	216	1.6
Infected (9 h light side)	11,059	92	0.83

Cells prelabelled with ^{14}C -TdR were infected or mock infected and pulse labelled with ^3H -TdR and centrifuged as described in Fig. 3. Samples (20 μl) were taken to determine the ^{14}C -labelled radioactive peak. The gradient of DNA from infected cells was then divided so that 54% and 46% of the ^{14}C -label from the left and right sides (referred to as heavy and light sides, respectively) were pooled. The percentages for the control gradient were 63 and 37, respectively. The pooled fractions were dialysed extensively against $0.1 \times \text{SSC}$. Hybridisation to cold viral DNA immobilised on nitrocellulose membrane filters (1 μg per filter) in duplicate was at 66°C for 24 h. Radioactivity in each filter was then determined after extensive rinsing to remove the unbound DNA.

The shift in density appears by 7 h after infection (Fig. 3) and increases further with time. It is interesting that the percentage of inhibition of DNA synthesis (Fig. 3 inset) also increases with time.

The buoyant density of ad 12 DNA is slightly higher than that of host cell DNA (cellular DNA, $\rho = 1.701$; ad 12 DNA, $\rho = 1.709$)¹. The observed shift in density, however, cannot be due to contamination with newly synthesised viral DNA as shown by Table 1. No viral DNA is detectable by hybridising the newly synthesised DNA pooled from the heavy or light side of CsCl gradients to unlabelled viral DNA immobilised on filters. This is consistent with the earlier report that the onset of viral DNA synthesis is approximately 12 h after infection in these cells⁹.

The genome of most higher organisms contains classes of DNA with different reiteration frequencies⁸. To determine whether infection affects the synthesis of classes of DNA with different degrees of repetition, the kinetics of reassociation of newly synthesised DNA from both infected and

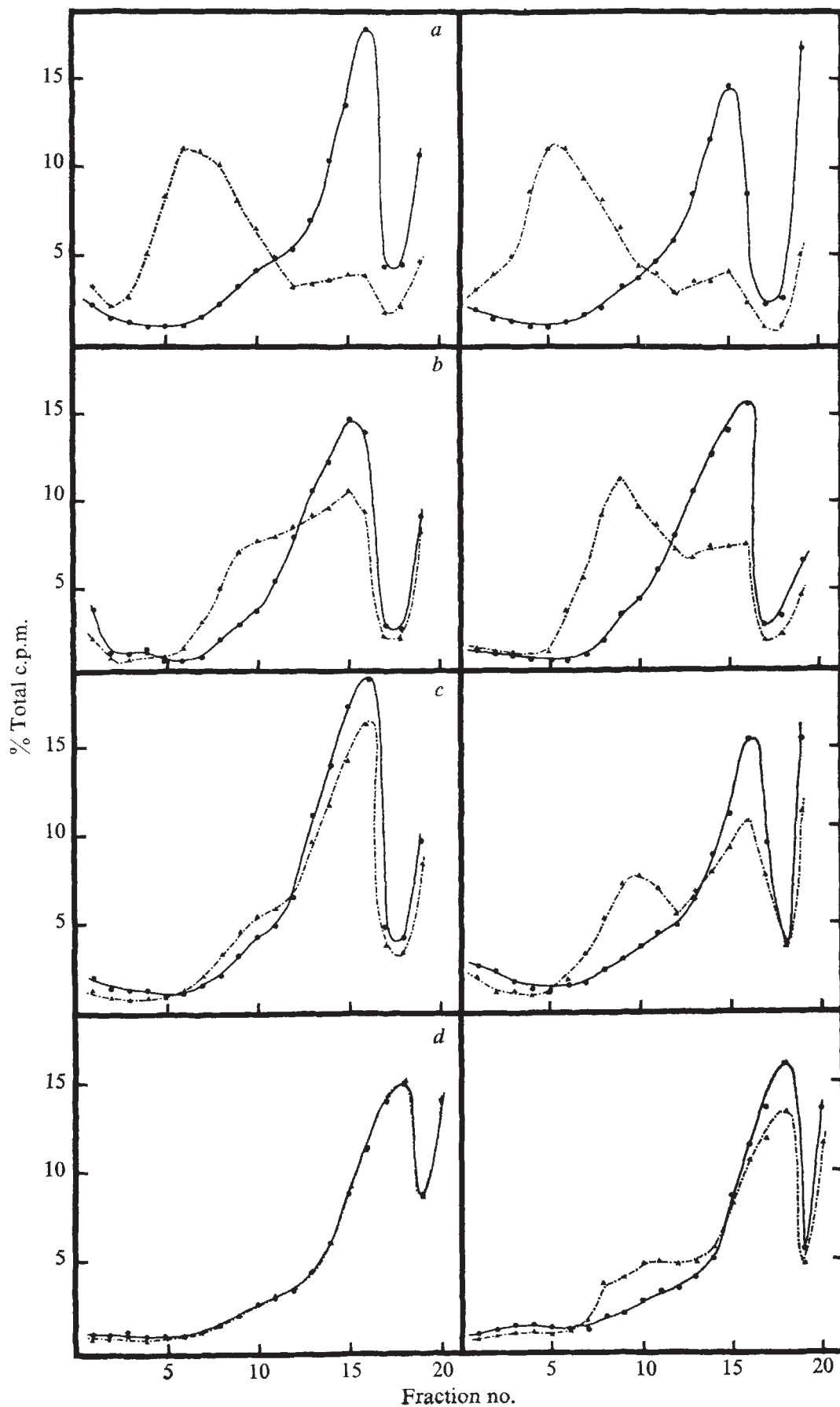


Fig. 1 Human KB cells, grown on monolayers and prelabelled with ^{14}C -TdR ($50 \mu\text{Ci mmol}^{-1}$, New England Nuclear Corp.) for 15–20 h, were trypsinised and resuspended in infecting medium (Joklik + 1% calf serum). About 3×10^6 cells in 1.5 ml were seeded in 25 cm^2 flasks and adsorbed with 3×10^3 virus particles per cell by shaking at 37°C . Control cells were treated similarly except without the virus. After 90 min of adsorption, 3.5 ml of growth medium (α -MEM, Flow Laboratories) containing 10% serum was added and the cells were incubated at 37°C . Eight hours after infection, ^3H -TdR (5 Ci mmol^{-1} , New England Nuclear Corp.) was added for a 15 min pulse and chased for the indicated times. Centrifugation of cellular DNA in alkaline sucrose gradients was at 20,000 r.p.m. for 6.5 h at 20°C using an SW 27.1 rotor in a Beckman ultracentrifuge. $\bullet$, ^{14}C ; $\blacktriangle$, ^3H . (Direction of sedimentation from left to right.) Left, control; right, infected; *a*, 0.25 h pulse; *b*, 2.5 h chase; *c*, 4 h chase; *d*, 6 h chase.

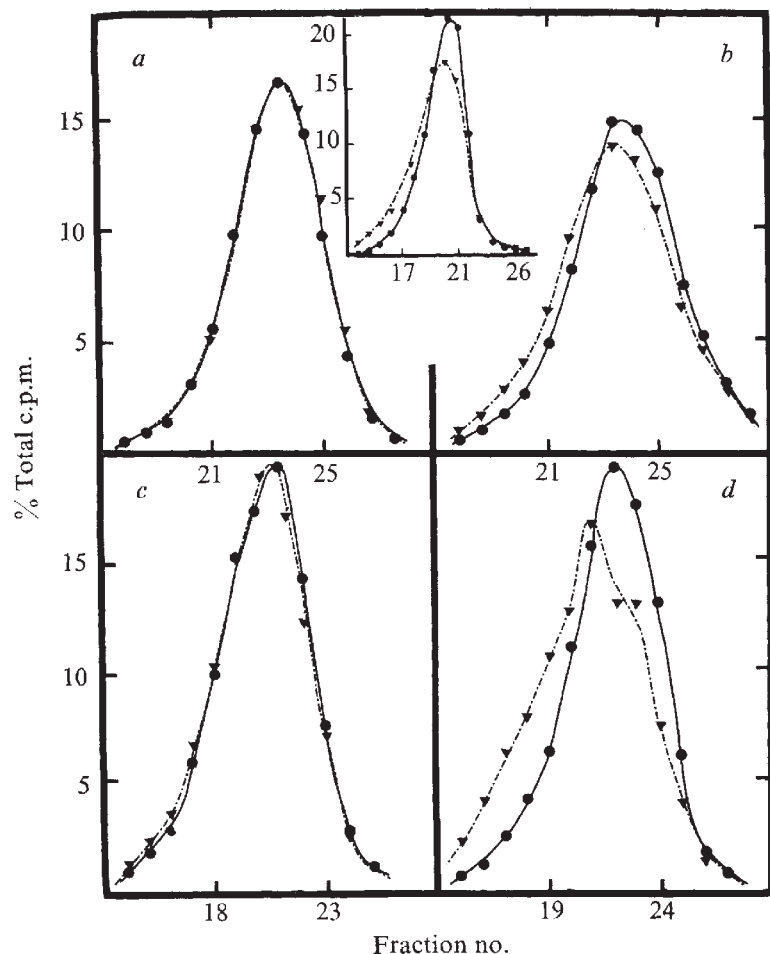


Fig. 2 Cells were prelabelled and infected as described in Fig. 1. They were pulse labelled for 1 h with ^3H -TdR and washed and lysed in 4 ml of phosphate buffer (0.1 M NaH_2PO_4 , 0.001 M EDTA, 0.1% sodium dodecyl sulphate (SDS), adjusted to pH 6 by adding 1 M Na_2HPO_4) and treated with Pronase (1g ml^{-1}) for 30 min at 37°C . For the inset, DNA was further purified by phenol extraction, dialysing extensively against Tris buffer (0.01 M, pH 7.3), and then treating with S_1 nuclease. Tris buffer (1 M, 0.46 ml) containing 10% sarkosyl was added to adjust the final concentration to 0.1 M Tris—1% sarkosyl, pH 7.3. DNA was sheared to an average molecular weight of 2×10^7 daltons by passing the solution through a 21-gauge needle. CsCl powder (5.4 g) was added to the solution in a siliconised polyallomer tube and then overlaid with mineral oil. Centrifugation was in a 50 titanium rotor at 40,000 r.p.m. at 20°C for 65 h. Ten-drop fractions were collected and radioactivity in each fraction was determined after cold TCA precipitation. $\bullet$, ^{14}C ; $\blacktriangle$, ^3H . a, control; b, infected; c, control, labelled with deoxycytidine; d, infected, labelled with deoxycytidine. (Direction of sedimentation from right to left.)

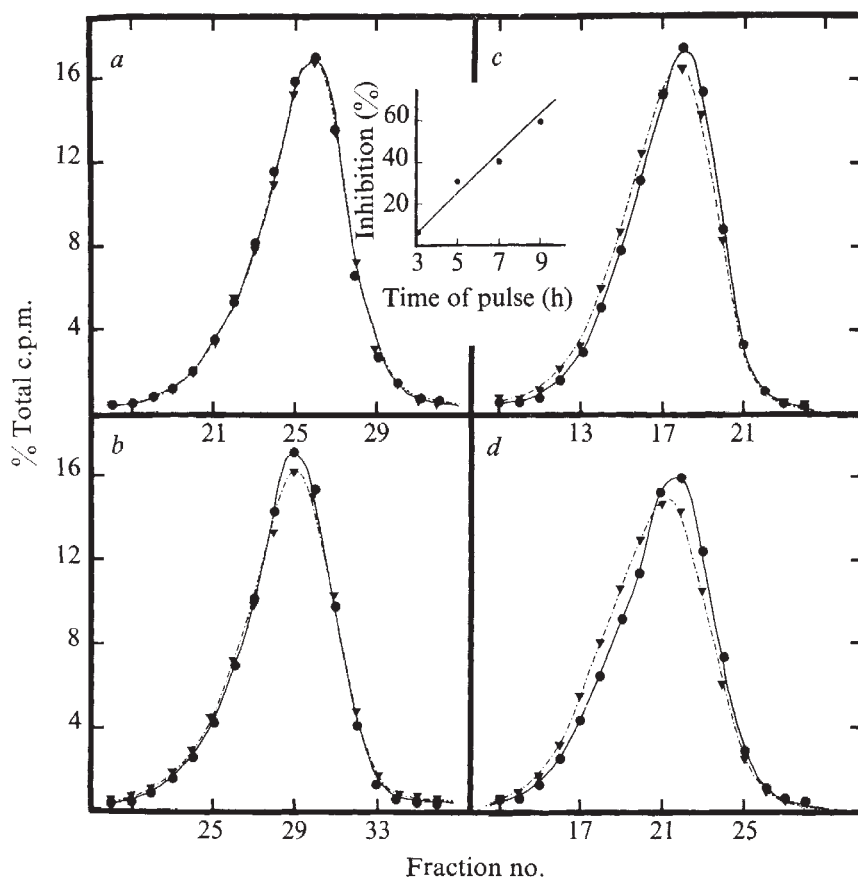


Fig. 3 Cells, prelabelled with ^{14}C -TdR, were infected or mock infected as described in Fig. 1. They were then pulse labelled with ^3H -TdR for 30 min at 3(a), 5(b), 7(c) and 9 h(d) after infection. DNA was centrifuged to equilibrium in CsCl as described in Fig. 2. Percentage of inhibition of cell DNA synthesis (inset) was determined by comparing the ratio of ^3H -DNA to ^{14}C -DNA of the control cells to that of infected cells.

uninfected cells was studied. KB cells were infected with ad 12 and then labelled with ^3H -TdR for 2 h at 7 h after infection. Control cells were labelled with ^{14}C -TdR. The two were combined and mixed with unlabelled, uninfected cells in order to drive the reannealing reaction by excess cold DNA. The reassociation of denatured DNA was followed using the technique of S_1 nuclease digestion of single-stranded DNA^{7,8}. As Fig. 4 shows, the reassociation of DNA follows a multi-component pattern with an apparent break at C_{ot} of 10. Only 20% of the newly synthesised DNA from uninfected cells reassociates with a C_{ot} of 10, whereas 30% of the DNA synthesised in the infected cells reassociates with the same C_{ot} value. Thus, the repetitive sequences represent a greater percentage of newly synthesised DNA in the infected cells.

In human KB cells the DNA synthesised in a short pulse is mostly in the form of small fragments. The conversion of these fragments into high molecular weight DNA is slower in cells infected with ad 12. This slow rate of chain elongation could be due to an inhibition of cell DNA synthesis (inset Fig. 3), since a similar result is obtained when DNA synthesis is inhibited by treating these cells with excess thymidine (our unpublished observation).

Selective inhibition of cell DNA synthesis after infection by ad 12 is the first report of such an effect by an animal virus. The biological significance of this selective inhibition is unknown. Integration of viral DNA into the DNA of these cells has been detected later in infection⁹. It is possible that those classes of DNA which are selectively synthesised are the sites for the integration of viral DNA.

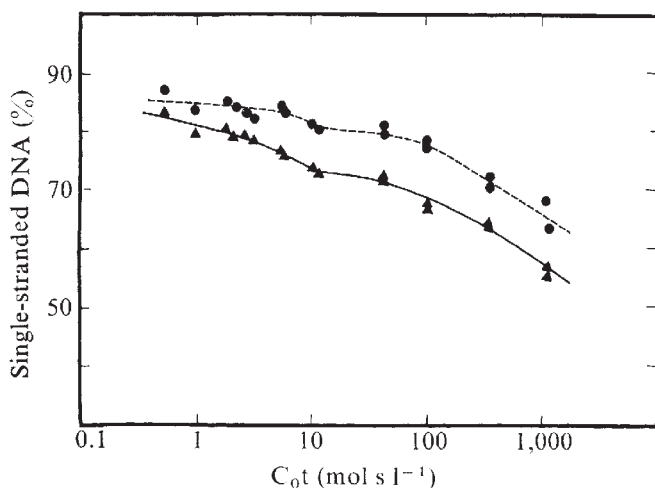


Fig. 4 About 6×10^8 cells were infected or mock infected as described in Fig. 1. After 7 h of infection, the cells were labelled with ^3H -TdR for 2 h. Control cells were labelled with ^{14}C -TdR. The two were combined and mixed with 6×10^8 unlabelled cells and lysed in Tris-SDS buffer (0.01 M Tris, 0.1% SDS). After digestion with Pronase (1 g ml⁻¹) at 37 °C, DNA was phenol extracted and ethanol precipitated. After resuspension in distilled water, $1 \times \text{SSC}$ (0.15 M NaCl, 0.015 M sodium citrate) was added to a final concentration of $0.1 \times \text{SSC}$. RNase treatment (50 $\mu\text{g ml}^{-1}$) was for 30 min at 37 °C. After phenol extraction, the DNA was ethanol precipitated and redissolved in 0.1 M Tris, pH 7.3 and dialysed extensively against the same buffer. ^3H -DNA was sonicated (the size of sonicated DNA was the same as sonicated ^{14}C -DNA as shown by their cosedimentation in alkaline sucrose gradients) chelex treated, and denatured by boiling. NaCl was added to a final concentration of 0.3 M. Reassociation was in a 2 ml siliconised flask at 67 °C. Samples (50 μl) were taken and treated (untreated as input) with S_1 nuclease in the presence of denatured calf thymus DNA (20 $\mu\text{g ml}^{-1}$) at 37 °C for 30 min. DNA resistant to S_1 was precipitated with cold trichloroacetic acid (TCA). DNA concentration was 1.45 mg ml⁻¹. Specific activity of ^3H -DNA was 1.4×10^4 c.p.m. mg⁻¹ and that of ^{14}C -DNA was 4.7×10^3 c.p.m. mg⁻¹. Resistance of denatured DNA to S_1 nuclease was 5% for ^3H -DNA and 4% for ^{14}C -DNA. Resistance of non-denatured DNA was 95% for both ^3H -DNA and ^{14}C -DNA. ●, ^{14}C ; ▲, ^3H .

The mechanism by which the selective inhibition of cell DNA synthesis is induced is unclear. It is possible that infection results in the selective inhibition of host DNA polymerases which may have specificity with respect to the recognition of their templates. But the direct role of the functional genes of the virus cannot be excluded, especially since no shift in density is observed if the cells are infected with ultraviolet-irradiated ad 12 (M.M.P. and S.M., unpublished). Genetic analysis has shown the presence of two¹⁰ or three¹¹ complementation groups which are necessary for the replication of viral DNA. These could be genes coding for viral specific polymerases. Selective inhibition of cell DNA synthesis could be due to recognition of certain regions of host DNA by such postulated polymerases.

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Are glycoproteins and glycosaminoglycans components of the eukaryotic genome?

THE eukaryotic genome is composed primarily of DNA and a complex and heterogeneous array of proteins. Evidence is rapidly accumulating to suggest that these chromosomal proteins are important in determining structural as well as functional properties of the genome^{1–6}. In addition to being implicated in packaging of the cell's genetic information^{7–12}, chromosomal proteins render limited and defined genetic sequences available for transcription^{13–23}. But the specific properties and mode of interaction of genome components remain to be resolved. We present here evidence that several molecular weight classes of chromosomal proteins are glycoproteins. Furthermore, we report the association of glycosaminoglycans, another class of carbohydrate-containing macromolecules, with the eukaryotic genome.

Exponentially growing HeLa S_3 cells were labelled for 48 h with ^3H -glucosamine (1 $\mu\text{Ci ml}^{-1}$) and three nuclear preparations were isolated. Nuclei containing intact nuclear envelopes as well as some adhering cytoplasm (crude nuclei) were prepared by osmotically swelling cells in 10 mM KCl, 1.5 mM MgCl_2 and 10 mM Tris, pH 7.4, followed by lysis in a Dounce type homogeniser. Nuclei stripped of the outer aspect of the nuclear envelope (clean nuclei) were prepared by washing crude nuclei with the non-ionic detergent Triton X-100. Chromatin was prepared from clean nuclei as reported before²⁴. The incorporation of ^3H -glucosamine into various molecular weight classes of proteins from the three nuclear preparations was determined by electrophoretic fractionation according to molecular weight in sodium dodecyl sulphate (SDS)–polyacrylamide gels.

Significant levels of incorporation of ^3H -glucosamine into chromatin-associated non-histone polypeptides which migrate

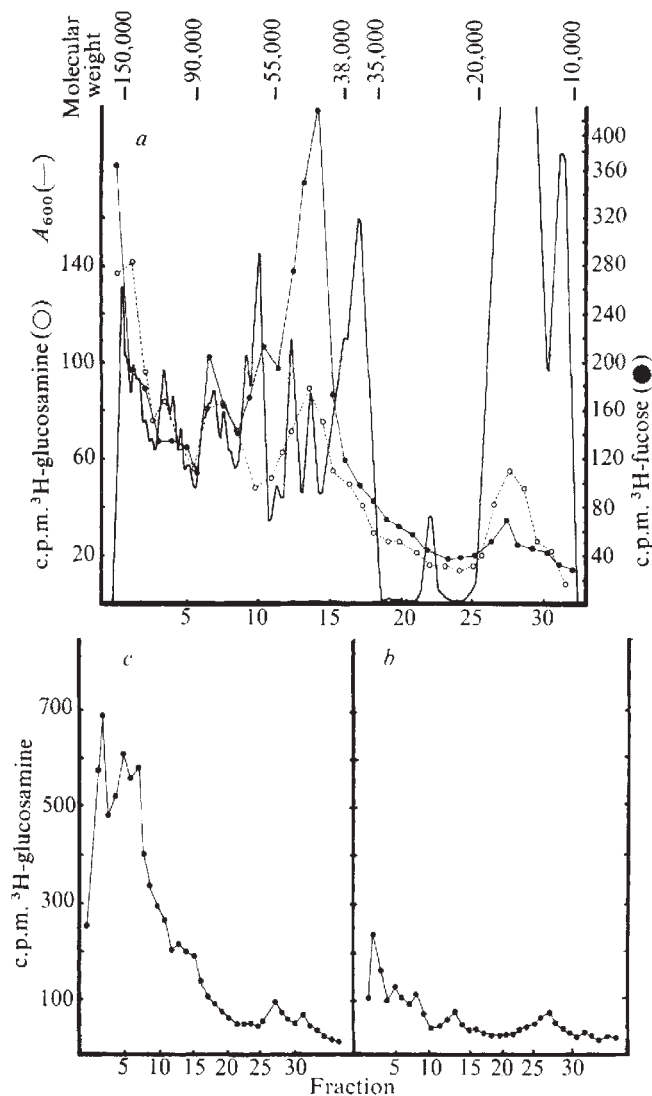


Fig. 1 SDS-polyacrylamide gel electrophoretic fractionation of proteins from chromatin (a), clean nuclei (b) and crude nuclei (c). Nuclear material was dissociated in 1% SDS, 1% β -mercaptoethanol and 0.01 M sodium phosphate, pH 7.0, and dialysed against 0.1% SDS, 0.1% β -mercaptoethanol and 0.01 M sodium phosphate, pH 7.0. Electrophoresis was carried out as described before in 0.6×7.5 cm, 7.5% polyacrylamide gels with 0.6×2 cm, 2.3% stacking gel²⁵.

in the 150,000–90,000 (fractions 1–6), 90,000–55,000 (fractions 7–10), and 55,000–38,000 (fractions 11–16) molecular weight regions of SDS-polyacrylamide gels are evident (Fig. 1a). Incorporation of ^3H -glucosamine is also observed in the 10,000–15,000 molecular weight region of the gel (fractions 26–30), where histone polypeptides migrate. But since tryptophan-containing non-histone chromosomal proteins also migrate in the 10,000–15,000 molecular weight region of these gels, our data do not establish unambiguously whether these ^3H -glucosamine containing polypeptides are histones or low molecular weight non-histone chromosomal proteins.

When chromosomal proteins from cells labelled with ^3H -glucosamine were subjected to acid hydrolysis²⁵ followed by paper chromatography²⁶, the ^3H was found primarily in the glucosamine and galactosamine, eliminating the possibility that the ^3H -glucosamine was metabolised to components other than amino sugars. Further evidence that glycoproteins are genome components is provided by the incorporation of ^3H -L-fucose into chromosomal polypeptides (Fig. 1a). Although ^3H -fucose is incorporated primarily into the same molecular weight classes of chromosomal proteins as those which incorporate ^3H -

glucosamine, there are pronounced differences in the relative extents to which the two labelled precursors are incorporated. This suggests variations of the carbohydrate components of specific molecular weight classes of chromosomal proteins. The ^3H -glucosamine and ^3H -fucose containing materials which migrate in a heterodisperse manner in SDS-polyacrylamide gels are degraded in the presence of Pronase, confirming that the carbohydrate groups are associated with proteins and not with other types of macromolecules.

Several approaches were used to establish that the glycoproteins associated with chromatin are *bona fide* genome components rather than membrane material which becomes associated with chromatin during the isolation process. When trypsinates (material removed from cells by treatment with 0.25% trypsin for 15 min in Hanks' balanced salt solution) from cells labelled with ^3H -glucosamine were added to unlabelled HeLa cells before chromatin isolation, no significant levels of radioactivity were recovered with the chromatin preparations. Similarly, when isolated plasma membranes²⁷ prepared from cells labelled with ^3H -glucosamine were added to unlabelled HeLa cells before chromatin isolation, there were no significant levels of radioactivity in the chromatin. Together these results indicate that the labelled macromolecules associated with our chromatin preparations are not contaminating cell surface material. Figure 1b and c shows the SDS-polyacrylamide gel electrophoretic radioactivity profiles of ^3H -glucosamine-labelled polypeptides of crude nuclei and clean nuclei. While comparison of the electrophoretic profiles of these nuclear preparations with that of chromatin suggests a similar distribution of glycoproteins in the low molecular weight regions of the gels (10,000–35,000), significant quantitative, and perhaps qualitative, variations are evident in the higher molecular weight regions.

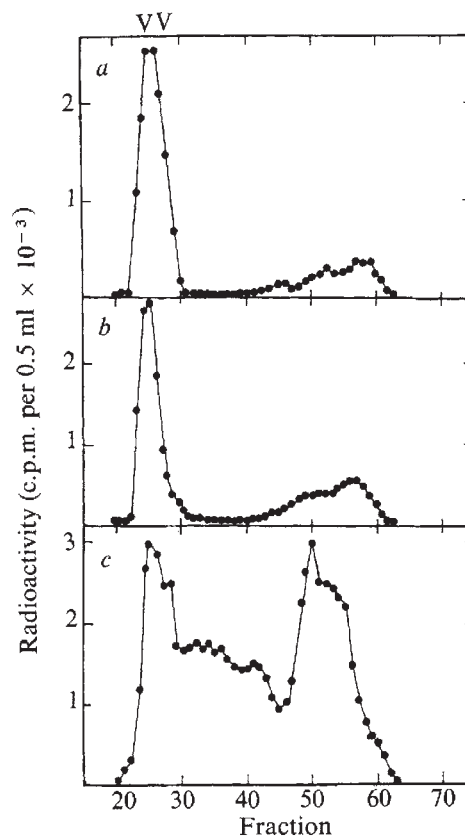


Fig. 2 Sephadex G-50 profile of Pronase-digested ^3H -glucosamine labelled nuclear material at different stages of purification: chromatin (a), clean nuclei (b), crude nuclei (c). Samples were loaded on to a Sephadex G-50 column (124 cm $\times$ 1.5 cm) and eluted with 0.05 M Tris, pH 8.5. Fractions (5 ml) were collected. The void volume of the column (VV) is indicated.

Although these findings by no means conclusively eliminate the presence of nuclear membrane material in our chromatin preparations, the pronounced differences in the glycoproteins of nuclei and chromatin preclude the possibility that the glycoproteins associated with chromatin are random contaminants from the nuclear membrane. It should be noted that when chromatin is purified by centrifugation through 1.7 M sucrose, which should eliminate membrane material, more than 90% of the glycoproteins present in the chromatin preparation remain associated with the purified chromatin pellet. The polyacrylamide gel electrophoretic profiles of these chromatin preparations before and after sucrose sedimentation are indistinguishable. When ^3H -thymidine-labelled chromatin is subjected to this purification procedure, a similar distribution of DNA between the pellet and supernatant is observed. If selected membrane-derived glycoproteins are isolated with chromatin, these may represent specific sites of interaction between the genome and the nuclear membrane. The possibility should also be considered that carbohydrate-containing nuclear material—glycoproteins and glycosaminoglycans (discussed below)—constitutes components of the annulate lamellae²⁸ or nuclear pore complex²⁹⁻³¹.

Further characterisation of the carbohydrate-containing nuclear material labelled with ^3H -glucosamine was carried out as follows. Crude nuclei, clean nuclei, and chromatin were subjected to Pronase digestion to hydrolyse polypeptides, and Pronase-resistant material was analysed by Sephadex G-50 chromatography (Fig. 2). In each run the sample used originated from a comparable number of labelled cells. Clearly the material of high molecular weight, eluting at the void volume of the column, accompanied the chromatin throughout the purification procedure. Furthermore, there was little qualitative or quantitative difference between the clean nuclei and final chromatin preparations in terms of the distribution of radioactivity among different size fractions of glycopeptides. The original crude preparation of nuclei, however, contained considerably more materials which were partially or wholly included by the gel. The high molecular weight nature of the ^3H -glucosamine-labelled material from HeLa chromatin, which was excluded from the Sephadex G-50 column, was confirmed by chromatography on a BioGel P-100 column when it again eluted with the excluded volume. This carbohydrate-containing material was analysed further by ion exchange chromatography on a DEAE-cellulose column using a linear gradient of ammonium acetate as eluant^{32,33}. Most of the radioactivity eluted at salt concentrations (1.0–1.5 M) which characteristically elute chondroitin sulphates. Hyaluronic acid, a non-sulphated glycosaminoglycan, which elutes at approximately 0.6 M, was clearly not present. Since the high molecular weight ^3H -labelled material was hydrolysed both by hyaluronidase and by chondroitinase, and heparan sulphates are not, it is reasonable to conclude that the labelled material is a glycosaminoglycan in the chondroitin class. This conclusion is reinforced because acid hydrolysis of the labelled material releases a radioactive monosaccharide component with the chromatographic mobility of galactosamine. No radioactivity from this high molecular weight material was found in glucosamine, the amino sugar component of hyaluronic acid and heparan sulphates. Furthermore, the polymer was not degraded by nitrous acid³⁴.

Our results suggest that at least two classes of carbohydrate-containing materials are associated with the isolated genome—chromatin—of HeLa S₃ cells. Glycoproteins with molecular weights ranging between 10,000 and 190,000, as well as glycosaminoglycans of high molecular weight have been identified. The latter do not seem to migrate into SDS-electrophoretic gels in the conditions we used. Characterisation of glycosaminoglycans as components of the eukaryotic nucleus is consistent with a recent report by Bhavanandan and Davidson³⁵. Although the significance of our findings remains to be resolved, a role for glycoproteins and mucopolysaccharides in the structure or transcriptional and replicative functions of the genome can be considered.

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Different degrees of interspecies homology in immunoglobulin λ chain constant domain correlated with three-dimensional structure

It is generally accepted that evolution of proteins is a consequence of duplications and point mutations occurring in genes coding for them. Palaeogenetics of Pauling and Zuckerkandl¹ as well as protein phylogenetics introduced by Fitch and Margoliash² (and subsequently modified by other workers) assume that comparative analyses of amino acid sequence data in terms of mutational distances (be it minimal mutational distances, corrected mutational distances or accepted point mutations per 100 amino acids), inferred to exist among genes coding for them, yield a realistic image of evolution; thus, genealogical trees of proteins (that is, dendrograms of mutational distances) can be constructed which relate to main evolutionary events thought to occur in the past.

Alternatively, Epstein^{3,4} suggested that the requirement to conserve a correct tertiary structure is the main governing force of protein evolution, the point mutations being mere driving forces of the evolution (this is because "biological function is more a correlate of macromolecular geometry than

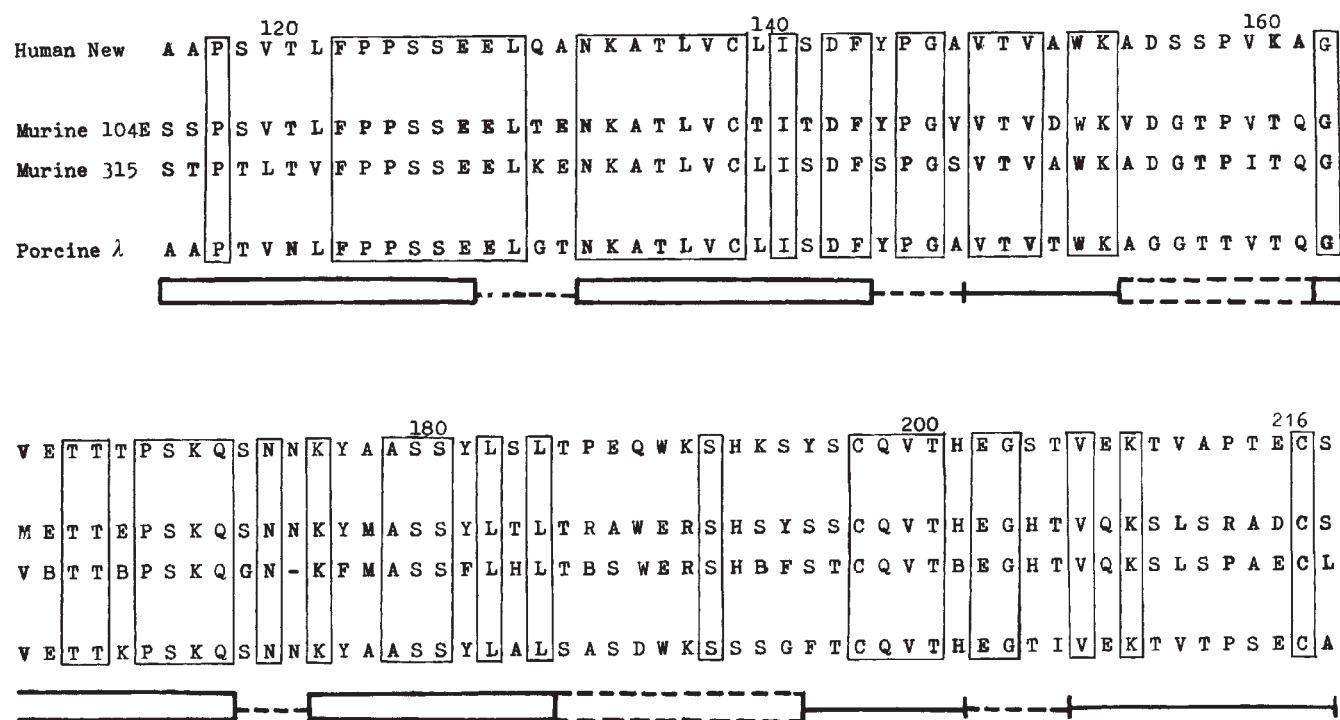


Fig. 1 Amino acid sequences of constant regions of immunoglobulin λ chains. Amino acid sequences are given in a one-letter notation for amino acid sequences²². Positions are numbered from the N-terminus. Sources of data: porcine normal λ chains¹²; human myeloma New¹³; murine myeloma 104E (ref. 14), murine myeloma 315 (ref. 15). The residues common to all sequences compared are indicated by a frame. Code for elements of the tertiary structure^{13,15}: —, four-segment layer of the antiparallel β pleated sheet; ---, three-segment layer of the antiparallel β pleated sheet; . . . , hairpin bends; - . - . , loops at the surface of the domain.

of chemical detail²⁵); according to this view, analysis of amino acid sequence data in terms of mutational distances are irrelevant⁶. Rather, the sequence data should be analysed in terms of (1) regions of primary structure which correspond to main elements of the three-dimensional structure (that is, α helices, β pleated sheets, β bends, and so on) and (2) constraints imposed by correct folding on free amino acid interchangeability in these regions, in relation to chemical homologies existing among side chains of amino acids under consideration⁷.

Most of the protein dendrograms fit palaeontological data well, seemingly supporting concepts of palaeogenetics and protein phylogenetics. On the other hand, results of crystallographic⁹ and model-building⁹ studies tend to agree with Epstein's view. Also, statistical analyses of codon variability carried out by Fitch and Markowitz¹⁰ and by Holmquist¹¹ do suggest that, in the course of evolution, (three-dimensional) constraints influence amino acid interchangeability in proteins in a decisive way. Obviously, further experimental data may provide better understanding of the problem.

We have determined the primary structure of the constant region (that is, a C-proximal half) of pig immunoglobulin λ chains¹². This structure is particularly suitable for evolutionary

studies: amino acid sequences of constant regions of human¹³ and murine^{14,15} λ chains are known, together with the three-dimensional structure of two human λ chains (New¹³ and Mcg¹⁶). The constant regions of these latter two chains fold independently from their variable regions (that is, N-proximal halves of the chain), representing an autonomous protein domain; the overall pattern as well as the details of the folding are almost identical in both the constant domains. The same is true for the constant domain of a mouse myeloma κ chain¹⁷ and it seems reasonable to assume that the same folding pattern exists in other mammalian species.

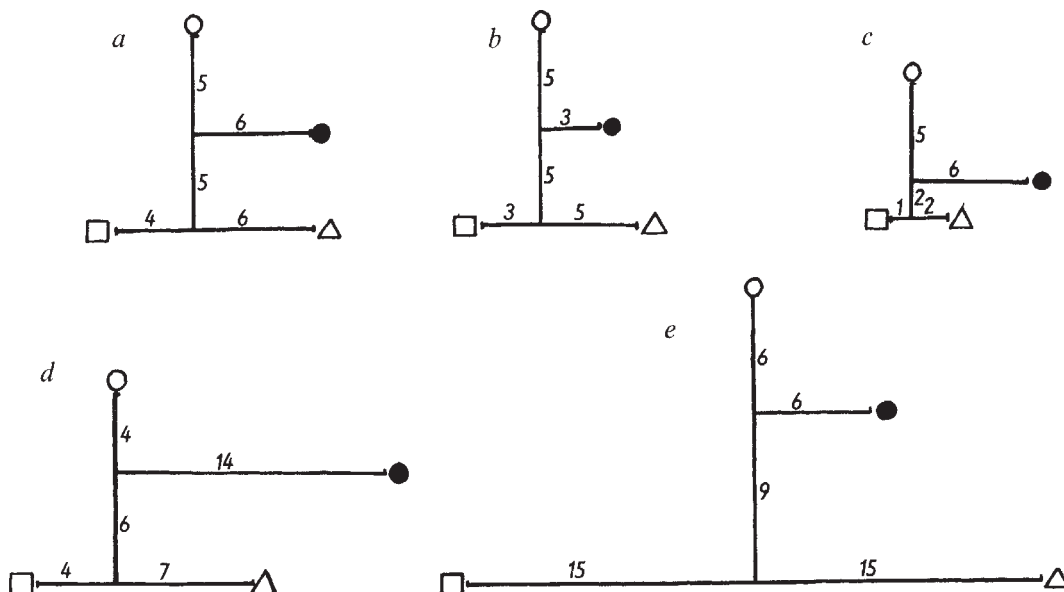
For comparison, we considered separately those segments of primary structure of λ chain constant domain which correspond to elements constituting the three-dimensional structure of the domain, namely, two layers of antiparallel β pleated sheets (the first one composed of four and the second of three polypeptide chain segments), four short hairpin bends and two loops at the surface of the domain (Fig. 1). The segments homologous in λ chains from different organisms were compared according to two alternative procedures. First, the number of identical amino acid residues was evaluated (Table 1). Second, from the number of amino acid replacements observed in one segment with

Table 1 Comparison of polypeptide chain segments from the constant region of immunoglobulin λ chains

	4 Segments of β -pleated sheet layer		3 Segments of β -pleated sheet layer		4 Hairpin bends		2 Surface loops		Whole domain	
	A	B	A	B	A	B	A	B	A	B
Mouse 104E-Mouse 315	75	10	75	8	67	18	67	13	71	11
Pig-Mouse 104E	82	9	54	15	67	18	39	31	67	16
Pig-Mouse 315	80	10	62	14	47	29	39	30	64	17
Pig-Man	95	3	75	8	73	11	33	30	75	10
Man-Mouse 104E	87	8	62	12	73	13	33	30	69	14
Man-Mouse 315	82	10	62	11	53	22	33	31	63	16
Range	75-95	3-10	54-75	8-15	47-73	11-29	33-67	13-30	63-75	11-17
% Identical amino acid residues in all sequences compared	67		50		47		6		51	

A, Percentage identical amino acid residues in this category of segments; B, minimal mutational distance per 100 nucleotides.

Fig. 2 Dendrograms constructed from mutational distances of segments of λ chain constant domain homologous in different organisms. *a*, Whole domain; *b*, three-segment layer of the β pleated sheet; *c*, four-segment layer of the β pleated sheet; *d*, hairpin bends; *e*, surface loops. Numbers represent mutational distances per 100 nucleotides. $\square$, Man; $\triangle$, pig; $\circ$, mouse 104E; $\bullet$, mouse 315.



respect to the other one, minimal mutational distance² of that pair was calculated (Table 1). The mutational distances were used to construct dendrograms (Fig. 2).

By the first procedure, segments involved in non-covalent interactions of the domain core (four-segment layer of the β -pleated sheet and, to a lesser extent, three-segment layer of the β -pleated sheet as well as the hairpin loops) were found to be highly conservative in terms of amino acid substitutions, whereas segments which are exposed to the surface of the domain and only marginally participate in the folding architecture (surface loops) are much less conservative (Table 1).

The second procedure shows that segments corresponding to different elements of the three-dimensional structure accumulate mutations at a different rate determined by their particular folding constraints ("conservation laws"), the constraints causing the accepted amino acid substitutions to be non-random events¹⁸. Accordingly, the surface loops, being subject to only faint constraints, display a dendrogram which approaches the phylogenetic tree of the species derived from palaeontological data. The same is true for the dendrogram of the whole domain. In contrast, the four-segment layer of the β -pleated sheet, being a subject to very strong constraints, displays a dendrogram with quite unnatural and distorted "evolutionary distances". For example, the (intraspecies) mutational distance between mouse λ chain subtypes would be, according to this dendrogram, almost six times longer than the (interspecies) distance between pig and man.

We thus conclude that the dendrograms constructed from mutational distances of short segments of primary structure (a procedure widely used among immunochemists) often lead to erroneous results. Figure 2 shows that only large regions of primary structure (such as the whole domain) or segments under exceptionally low folding constraints (such as surface loops) can serve for calculation of mutational distances which reasonably approximate the course of evolution. Our analysis also shows that protein genealogical trees (commonly thought to represent reconstructions of a time course of supposed evolutionary events) arise as consequences of "amino acid selection rules", that is, constraints imposed by folding requirements on free amino acid interchangeability in proteins¹⁹.

A large body of primary structures of immunoglobulin polypeptide chains has accumulated but theoretical studies based on these structures have mostly neglected the unique three-dimensional aspects of the "immunoglobulin fold", comparing the structure only from the point of view of underlying mutational distances. Taking into account folding peculiarities of immunoglobulins, a plausible explanation¹⁹⁻²¹

might be provided for some of the paradoxical features of antibody molecules.

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Human fibrinogen possesses binding site for staphylococci on A α and B β polypeptide chains

THE fibrinogen molecule, which comprises 5% of plasma proteins, is involved in enzymatic reactions responsible for formation and dissolution of the fibrin clot¹. Thereby, fibrinogen is being transformed by thrombin into fibrin, which is cross linked by glutamyltransamidase (factor XIII), and ultimately degraded by plasmin. The A α , B β and γ polypeptide chains (nomenclature according to the recommendations of the Committee on Nomenclature of the International Society on Thrombosis and Haemostasis, 1971) of fibrinogen involved in these reactions and the sites of enzymatic attack thereon are defined²⁻⁸. In addition to

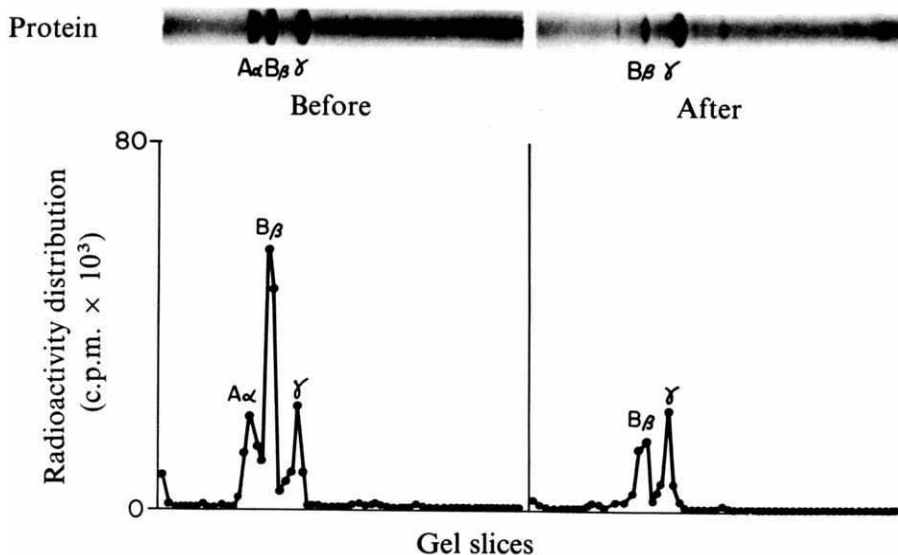


Fig. 1 Interaction of staphylococci with polypeptide chains of reduced and alkylated ^{125}I -human fibrinogen. Before interaction with staphylococci, three major bands representing $A\alpha$, $B\beta$ and γ chains and corresponding three radioactivity peaks could be distinguished. After interaction, the $A\alpha$ chain and part of $B\beta$ chain disappeared indicating their binding to staphylococci. γ Chain remained in solution. SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Weber and Osborn¹⁶. Gels were stained with Coomassie blue and sliced for radioactivity measurement.

being involved in the enzymatic reactions responsible for formation and dissolution of the fibrin clot, fibrinogen exhibits the ability to interact in a non-enzymatic reaction with pathogenic staphylococci to cause their clumping⁹. Clumping of staphylococci is used to detect fibrinogen and its derivatives in blood and body fluids^{10,11}. This reaction can detect as little as 0.03 μg human fibrinogen or its derivatives. The mechanism through which human fibrinogen interacts with staphylococci is however, unexplained and the localisation of the binding site(s) for staphylococci remains unknown. We therefore studied localisation of the binding site for staphylococci on human fibrinogen. We now report that the binding site for staphylococci is located on $A\alpha$ and $B\beta$ chains of human fibrinogen.

We used human fibrinogen (Kabi) labelled with ^{125}I according to the method of McFarlane¹² and reduced and alkylated to have separate polypeptide chains¹³. A mixture of polypeptide chains of fibrinogen was incubated with staphylococci in the presence of 3 M urea which does not inhibit the clumping reaction^{14,15}. Staphylococci with bound subunits of fibrinogen were removed by centrifugation and the supernatant was analysed for residual, unbound material. As it is shown in Fig. 1, before interaction with staphylococci we could distinguish in SDS-polyacrylamide gel three major bands representing $A\alpha$, $B\beta$ and γ chains of human fibrinogen. Accordingly, the radioactivity pattern showed three peaks in the sliced gel.

After the interaction between reduced and alkylated fibrinogen and staphylococci, $A\alpha$ and part of $B\beta$ chains disappeared as shown by protein staining and radioactivity peaks in the sliced gel (Fig. 1). In contrast, the γ chain

remained in solution, indicating lack of its binding to staphylococci. To gain a more precise insight into the extent of binding of individual chains of fibrinogen with staphylococci, we measured the amount of radioactivity in the SDS-polyacrylamide gel before and after interaction with staphylococci and computed the percentage radioactivity bound to staphylococci. The results of three independently conducted experiments (mean $\pm$ s.d.) were as follows. When we took ^{125}I -human fibrinogen which was not reduced, $91.7 \pm 10.2\%$ of radioactivity was bound to staphylococci. After reduction and alkylation of ^{125}I fibrinogen $95.0 \pm 2.0\%$ of $A\alpha$ chain and $65.3 \pm 11.9\%$ of $B\beta$ chain interacted with staphylococci. In contrast, only $23 \pm 9.0\%$ of radioactivity associated with γ chain has disappeared from the solution after interaction with staphylococci. Thus, these experiments indicate the high affinity of $A\alpha$ chain of human fibrinogen for staphylococci and the lower affinity of $B\beta$ chain. γ Chain possesses very low, if any, affinity towards staphylococci.

The fact that $A\alpha$ and $B\beta$ chains of human fibrinogen possess the staphylococci-binding site raises the next question. Do individual chains of human fibrinogen possess one or more binding sites for staphylococci? To answer this question, we compared the binding and clumping of staphylococci by non-reduced and reduced fibrinogen. Whereas non-reduced fibrinogen demonstrated excellent correlation between binding (91.7%) and clumping (titre 1024), reduced fibrinogen showed only significant binding (60%). The clumping activity of reduced fibrinogen was greatly diminished (titre 0-2). This suggests that individual chains separated by reduction of fibrinogen cannot link two

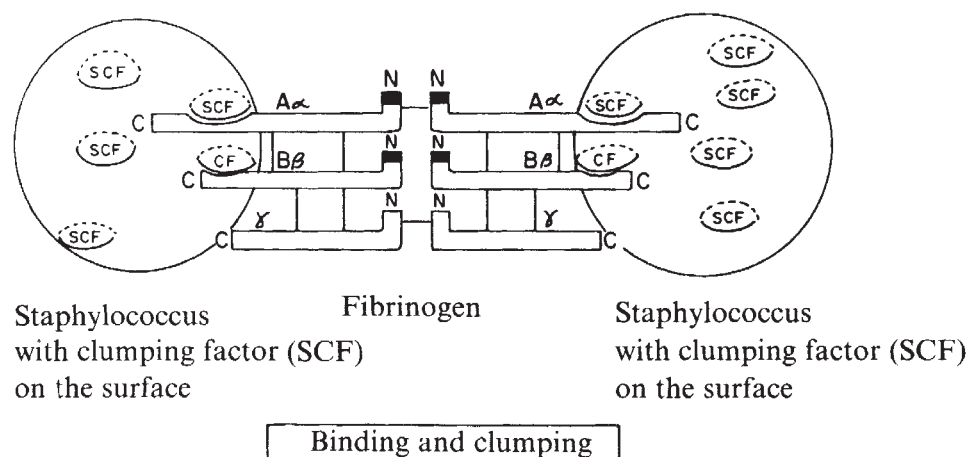


Fig. 2 Diagrammatic presentation of interaction between human fibrinogen and staphylococci. Binding site for staphylococcal clumping factor (SCF) is localised on $A\alpha$ and $B\beta$ chains between the N-terminal disulphide knot and the C-terminal end of the chains. At least two polypeptide chains with one binding site each need to be bound together by disulphide bridges to join two staphylococci. The dimeric structure of native fibrinogen molecule thus permits not only binding but also clumping of staphylococci.

or more staphylococci together to cause their clumping. A α and B β chains must be bound by disulphide bridges to clump staphylococci. Thus we can conclude that individual A α and B β chains possess only one binding site, since the clumping reaction, like most agglutination reactions, requires bivalent molecules which in this instance are represented by the dimeric structure of intact fibrinogen.

The following lines of evidence indicate that the binding site for staphylococci is located on the C-terminal portion of A α and B β chains of human fibrinogen. First, the binding sites were gradually destroyed during progressive degradation of the C-terminal portions of A α and B β chains with plasmin. Second, isolated fragment E (ref. 7) labelled with ^{125}I and isolated N-terminal disulphide knot obtained after CNBr-treatment of fibrinogen¹⁷ and labelled with ^{125}I did not bind or clump staphylococci (J.H., D.K.H., S.T. and A. Z. Budzynski, unpublished). Since both the N-terminal disulphide knot and fragment E represent that segment of the fibrinogen molecule which is devoid of the C-terminal portion of polypeptide chains, the lack of binding and clumping of staphylococci suggests that the binding site is located on the missing C-terminal portion of A α and B β chains.

These data represent the initial demonstration that human fibrinogen possesses a binding site for staphylococci on A α and B β chains. The binding site is located between the N-terminal disulphide knot and the C-terminal end of A α and B β chains. The dimeric structure of the fibrinogen molecule facilitates not only binding but also clumping as shown in Fig. 2. When the fibrinogen molecule is reduced, individual A α and B β chains interact with staphylococci but there is no clumping of staphylococci. This is because a least two chains with one binding site each need to be bound together by disulphide bridges in order to be able to link two staphylococci.

Demonstration of the binding site for staphylococci on A α and B β chains as described herein explains the mechanism of the clumping of staphylococci by human fibrinogen and its derivatives. It also offers a new approach to study the structure and the function of subunits of fibrinogen molecule. By using staphylococci it is possible to selectively absorb individual subunits of human fibrinogen. Similarly, our findings that the binding site for staphylococci is located beyond that region of the fibrinogen molecule from which fibrinopeptides are released or which participates in the polymerisation step, provides the basis for use of staphylococci to measure abnormal variants of fibrinogen. Furthermore, fibrinogen has been reported to have a role in intravascular immunological disorders¹⁸ which can be triggered by some microbial agents. The interaction between human fibrinogen and staphylococci may serve as an example of non-enzymatic reaction involving fibrinogen and possibly leading to its deposition around microbial antigens as postulated in some immune complex diseases. This function of clottable protein to interact with foreign particles in the environment of blood is phylogenetically an old phenomenon occurring in primitive species possessing clottable protein confined within haemocytes¹⁹. Preservation of the binding site for staphylococci on several mammalian fibrinogens studied²⁰ indicates that this property has been quite constant during mammalian evolution.

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Induced changes in gene conversion spectra

IN crosses, segregation at a heterozygous site may produce a low frequency of octads with spore ratios of 6+:2mutant or 2+:6m (meiotic segregation), or 5+:3m or 3+:5m (postmeiotic segregation), instead of the usual 4+:4m ratio. These irregular (aberrant) segregations are usually attributed to gene conversion involving hybrid DNA formation at the heterozygous site, with or without enzymatic correction of the resulting base mispairs. If the frequency and direction (to + and to m) of correction depend on the chemical nature of the mispair, then the chemical origin of the mutation involved in the mispair could affect its conversion spectrum, providing evidence in favour of hybrid DNA recombination models.

Leblon¹ showed a clear relationship between the molecular origin of induced mutations and their conversion spectra. He defined conversion spectra by two criteria—the frequency of postmeiotic segregation, and the relative frequencies of conversion to the mutant allele or to wild type. Four main kinds of spectrum were recognised and, by the use of specific mutagens and reversion tests, were identified with different kinds of molecular mutation. The studies reported here show, however, that the conversion spectrum for a single mutation can vary with genetic and environmental factors, thus a particular mutation giving one type of spectrum under one set of conditions may give a different type of spectrum under other conditions, although the mutation itself is unaltered.

The spectrum types and their associated kinds of mutation given by Leblon¹ were: type A, deletion frame-shifts, rare postmeiotic segregation and excess of conversion to wild type; type B, addition frame-shifts, rare postmeiotic segregation and excess of conversion to mutant; type C, base substitutions, postmeiotic segregation not rare, excess of conversion to wild type; type D, base substitutions, postmeiotic segregation not rare, excess of conversion to mutant.

We have used Pasadena strains of *Ascochola immersus*, with methods, controls for repeatability, phenocopies, new mutations and false spore-clusters as described before²⁻⁴. w-10 and w-78 are very closely linked, possibly allelic, spontaneous white ascospore mutations. The two original mutants gave 2-4% conversion in crosses to wild type, while certain strains isolated from 2+:6w octads in such crosses gave much higher conversion frequencies, 8-20% (refs 2 and 4). The reisolated mutants, referred to as HCF (high conversion frequency) strains, are isoelectrical

Table 1 Variation in gene conversion parameters with: (1) genetic factors; (2) temperature, in + × w crosses of *Ascobolus immersus*

Cross	Temp. (°C)	Total octads	(a) 6+ :2w + 2+ :6w %	(b) 5+ :3w + 3+ :5w %	(c) 6+ :2w + 2+ :6w 5+ :3w + 3+ :5w %	(d) 6+ :2w 2+ :6w	(e) 5+ :3w 3+ :5w
LCF w-78 × P5	17.5	18,406	3.77	0.47	8.0	0.40	1.04
HCF w-78 × P5	17.5	2,832	9.00	1.13	8.0	1.60	1.69
LCF w-78 × 1WT3	17.5	7,637	2.13	0.24	8.9	1.01	1.40
HCF w-78 × 1WT3	17.5	2,958	8.01	0.65	12.3	2.48	0.59
LCF w-10 × P5	17.5	18,034	3.25	0.31	10.5	0.64	0.72
HCF w-10 × P5	17.5	55,418	13.48	0.29	46.5	2.75	1.42
LCF w-10 × 81	17.5	5,570	4.45	0.71	6.3	1.21	0.25
HCF w-10 × 81	17.5	12,640	18.07	0.11	164.3	3.16	2.67
(ii)							
LCF w-78 × P5	10.0	23,503	0.99	0.40	2.5	0.35	3.70
	17.5	18,406	3.77	0.47	8.0	0.40	1.04
	22.5	5,418	2.69	0.29	9.1	2.40	0.61
HCF w-78 × P5	10.0	9,845	4.84	0.78	6.2	1.45	1.48
	17.5	2,832	9.00	1.13	8.0	1.60	1.69
	22.5	6,733	10.47	0.99	10.5	4.55	2.53
LCF w-10 × P5	10.0	4,853	1.23	0.16	6.7	0.13	7.00
	17.5	18,034	3.25	0.31	10.5	0.64	0.75
	22.5	11,328	2.22	0.21	10.5	3.05	0.50
HCF w-10 × P5	10.0	15,134	5.22	0.15	34.4	2.46	1.09
	17.5	53,230	13.55	0.31	44.8	2.76	1.40
	22.5	1,389	14.62	0.79	18.5	4.21	0.83

a, Total observed meiotic segregation frequency; b, total observed postmeiotic segregation frequency; c, ratio of observed meiotic and postmeiotic segregation; d and e, ratio of correction to wild type and to mutant in octads with (d) meiotic and (e) postmeiotic segregation.

with the original low converting (LCF) strains but differ from them in a very closely linked factor controlling their conversion frequency.

Data in Table 1i, comparing LCF and HCF crosses (of w-10 and w-78 to wild-type P5 and derived wild types 1WT3 or 81), show that gene conversion parameters varied with the genetic factor, which had major effects on conversion spectra as well as on conversion frequencies. Thus in crosses to P5, conversion was more often to mutant than to wild type in the LCF crosses, but the reverse was true in HCF crosses. The factor affected the absolute frequencies of observed meiotic and postmeiotic segregation, and also the frequencies of meiotic segregation relative to postmeiotic segregation. For example, in w-10 crosses with 81, meiotic segregation was six times more frequent than postmeiotic segregation in the LCF cross but was 164 times more frequent in the HCF cross.

Table 1ii shows that temperature also had major effects on conversion spectrum criteria. Thus for 6:2 and 2:6 octads in LCF crosses w-78 × P5 and w-10 × P5, conversion was much more often to mutant than to wild type at 10 °C and 17.5 °C but the reverse occurred at 22.5 °C. A similar reversal, with an opposite trend, is shown for the corresponding 5:3 and 3:5 octads. The absolute frequency of asci with postmeiotic segregation showed various changes with temperature; the relative frequencies of meiotic and postmeiotic segregation also changed; for example, for LCF w-78 × P5, from 2.46 at 10 °C to 9.13 at 22.5 °C. Many of these changes are significant at $P=0.01$ or 0.05 .

These effects of genetic and environmental factors on conversion spectra show that a given mutation may have one type of conversion spectrum (A, B, C or D, as defined by Leblon¹) in one set of circumstances but another kind in different conditions even though the mutation itself is unchanged. Thus LCF strains of w-78 and w-10 had spectrum type D at 10 °C and 17.5 °C, but type C at 22.5 °C. w-78 and w-10 LCF strains had spectrum type D at 10 °C and 17.5 °C but the corresponding HCF strains, carrying the same mutations, were of type C at those temperatures. The range 17.5 °C–22.5 °C is regarded as normal for making crosses of these Pasadena strains of *Ascobolus*, yet even within this narrow range the original mutants

(LCF strains of w-78 and w-10) had different types of conversion spectrum, D or C.

The spectrum criterion of relative frequencies of conversion to wild type and to mutant can thus be changed for a particular mutant (Table 1, columns d and e). The other criterion, the frequency of postmeiotic segregation, was less clearly defined by Leblon¹, being used on its own and also as a comparison of meiotic and postmeiotic segregation frequencies. Table 1, column c, shows that large changes in relative frequencies of meiotic and postmeiotic segregation can occur for a mutation.

Work with one standard set of environmental and genotypic conditions may therefore not reveal the range of conversion spectra which a single mutation can show. Because mutations may differ in their conversion responses to genetic and environmental factors^{4,5}, different mutants might have different conversion spectrum types in any single set of conditions, even if those mutations are of the same chemical type. In Leblon's data¹ (his paper 1, Tables 3–11) the differences between conversion spectrum types were clear for 72 out of 92 mutants, and in very distinctive cases qualitative changes of spectrum type by environmental or genotypic differences are presumably less likely than with the mutants used in our experiments.

Our results show that types of conversion spectrum for single mutations can vary with genetic and environmental factors. Deductions from conversion spectra alone about the molecular nature of any mutation should therefore be regarded as very tentative unless backed by evidence from mutation and reversion studies.

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reviews

Portrait of the philosopher

Asa Briggs

THIS huge biography* has many of the virtues and some of the defects of the huge nineteenth-century biographies which Lytton Strachey made fun of while at the same time ransacking them. It begins somewhat pretentiously and ends somewhat tendentiously, and the tone, not to speak of the style, is uneven throughout. There are far too many examples of "was to" and "would be". No Victorian biographer, of course, would have dealt as explicitly or at such length with Russell's sexual adventures as does Ronald Clark, but the treatment of these is not dissimilar from the way a Victorian would have dealt with them had he tried. There is a vast amount of unnecessary detail, and a fair amount of superfluous and repetitive commentary. Yet Clark is doubtless right to refuse to deal with Russell without revealing "the deep emotional complexities" of his personality. Russell himself—at least on one occasion—asked for a biographer who would not treat him as "a solemn stained glass saint existing only for purposes of edification". Like any Bloomsbury character—and such characters dominate many of the middle pages of this volume—he insisted, a little too indulgently, that a biographer would have to recognise above all that "I existed from my own centre."

It was a way of putting things which left a great deal out. It left out, for example, his Whig inheritance which could be identified more specifically than Clark does, and a corresponding set of Whig attitudes which he never entirely shook off. He used popular feelings when he chose to do so, but he never entirely shared them, and he knew instinctively, when he chose to do so, how to appeal to people with power. He was always interested in the relationship between freedom and power, and he never doubted that people should be listening to him on the subject. "I can understand your envying me," he once wrote to Nehru, "because a private person is not obliged to bear in mind the very com-



Bertrand Russell as a freshman at Trinity College, Cambridge

plex considerations which beset a head of state."

With Russell's Whiggery went a strong sense of the need for an intellectual aristocracy. "Surely one Darwin is more important than 30 million working men and women." The controversial Ralph Schoenman in a passage of admirable clarity, the kind of clarity which Russell himself often achieved and always appreciated, wrote that there were "serious intellectual doubts which inhibited him from engaging wholeheartedly in mass movements with revolutionary aims. He felt that cultural excellence and unique achievement were the product of favoured circumstance."

Russell's own unique achievement is dealt with fairly in this biography. He

turned to mathematics as a boy with delight, quickly choosing the most abstract parts" which he understood best. He needed to detach himself in order to pursue mathematical studies. "I like mathematics," he wrote, "because it is not human and has nothing particular to do with this planet or with the whole accidental universe—because, like Spinoza's God, it won't love us in return." Although he wrote and published *German Social Democracy* before *A Critical Exposition of the Philosophy of Leibniz*, he was stimulated and challenged more by Frege than by Bebel. The intellectual collaboration with Whitehead, which culminated in *Principia Mathematica*, is dealt with fully by Clark: so, too, less fully, is Russell's relationship with Wittgenstein. There is a great deal of relevant unpublished source material in the Bertrand Russell Archives at McMaster University, which Clark has carefully studied, and he has no difficulty in showing in what areas Russell pioneered and on what issues he himself changed his mind. All the references are useful, even when they are not conclusive. There is far more to be written, however, possibly in essays, about Russell's influence on other philosophers and on the study of philosophy itself. Indeed, long though this biography is, the author offers us no final reflections on Russell's position as a mathematician and philosopher.

It may well be that Russell learned more from continental European philosophy than he contributed to it, and that when we seek to establish the intellectual and cultural contours of this century his own thought will be related primarily to the intellectual and cultural history of this country and of the US. When he received the Nobel Prize it was for literature, and his own *History of Western Philosophy*, a best seller from the moment of its publication, must be judged in very different terms from *Principia Mathematica*.

Of his eminence—a Victorian concept—there was no doubt. Nor did he himself doubt it. Walking with a friend in Trinity, Cambridge, one day, he pointed to the marble busts on pedestals, waved his hand as he passed the last one, and said, "I shall be here one day." □

**The Life of Bertrand Russell*. By Ronald W. Clark. Pp. 766. (Jonathan Cape and Weidenfeld and Nicolson: London, 1975.) £6.95.

Controlled drug use

Concepts in Biochemical Pharmacology, Part 3. Edited by J. R. Gillette and J. R. Mitchell. (Handbook of Experimental Pharmacology, New Series, Vol. 28/3.) Pp. xxviii+480. (Springer: Berlin and New York, 1975.) DM 198; \$81.40.

MANY powerful drugs are now available and if they are to be used to best effect we need knowledge not only of how they act on the body, but also of how the body deals with the drug; both aspects are equally important in deciding how we use the drug. Important questions are: how much do we give? When? How? And to whom? This book is concerned with answering those questions. Work in this important branch of pharmacology has greatly expanded in the past decade, and although several texts have been published none are quite as detailed, or complete, as the volume of which this book constitutes the final part. In contrast to the two earlier parts, which were concerned more with methodology and general principles, this deals in detail with practical applications. Throughout, the editors have sought contributions from experts who have generally succeeded in combining the basic concepts with considerations of

their clinical implications.

The first two chapters deal rigorously but comprehensively with the mathematics of drug disposition. The second in particular relates the various models which have been developed to what actually happens in the body, with a full discussion of the importance of regional blood flow and protein binding. Subsequent chapters analyse factors which lead to individual variation in drug disposition (age, disease and genetic factors) and to variation within an individual (the route of drug administration and the form in which the drug is given). A section on drug interactions starts with a clear discussion of possible mechanisms and includes two interesting chapters which cover areas where beneficial interactions may occur: cancer and antimicrobial chemotherapy. The disadvantages of using antibiotics in combination are also stressed. A final section deals with some aspects of drug toxicity, particularly the role of chemically reactive drug metabolites in causing tissue damage.

The book achieves the same high standards of both content and presentation as its companion parts and together they constitute a most valuable reference work. Its detailed and rigorous approach make it most suited to clinical pharmacologists and

research workers, particularly those involved in drug development. At the same time, a lot of what it has to say could be directly translated into prescribing practice, and it is to be hoped that it will reach a much wider readership among the medical profession.

D. G. Haylett

Action of antitumour agents

Antineoplastic and Immunosuppressive Agents. Part 1 and 2. (Handbook of Experimental Pharmacology, New Series. Vol. 38/1 and 2.) Edited by Alan C. Sartorelli and David G. Johns. Vol. 1, Pp. xxiv+762; DM258; \$105.30. Vol. 2, Pp. xxxii+1067; DM 278; \$119.80. (Springer: Berlin and New York, 1975.)

THIS two-volume book comprises a series of reviews, 79 in all, which give as wide a coverage of the nature of antitumour agents and their action as has yet been seen in one work.

The general title might suggest that antineoplastic agents and immunosuppressive agents are different groups, whereas the latter are in practice drawn from the former. Only three chapters (Part I) are devoted specifically to immunosuppressive agents but the coverage is adequate; moreover, the immunological properties of agents are discussed in subsections in other chapters.

Part I contains the more general articles. It includes chapters on the rationale of drug design, the choice and use of agents, and drug resistance. The editors have also wisely included reviews on related topics: cell kinetics, cell growth regulation, radiation, immunotherapy, and pharmacology in the broadest sense (organism to molecule). Part II is devoted to a detailed treatment of various agents including hormones.

The team of well-known contributors assembled by the editors virtually ensured an authoritative compendium, packed with information, marked by a lack of uniformity in chapter organisation which should worry none but the perfectionist. Most of the contributions were probably written three or four years ago but a time lag is unavoidable in the preparation of such a work. New agents of real value do not emerge very often. It is therefore certain that those concerned with the fundamental aspects of drug action will find this handbook a valuable reference work for years to come. It is equally certain that the high price will almost totally exclude this meritorious work from private bookshelves.

J. A. Stock

Textbook of psychopharmacology

Psychopharmacology: a Biological approach. (Series in Experimental Psychology.) By Robert A. Levitt. Pp. x+502. (Hemisphere: Washington, June 1975. Distributed by Halsted Press.) £9.45.

THE world still needs honest jobbing textbooks, and it is within that genre that the present volume takes its place. In the introduction, Professor Levitt describes the origins of this text as being material he prepared for a graduate seminar: seven of his students became sufficiently enthused subsequently to help in the further development of certain chapters, and they now appear as authors or co-authors. The result of this admirably cooperative venture is a text which is business-like and straightforward, but at the same time has about it a special feeling of enthusiasm. One may suspect that in this particular set of seminars no-one fell asleep.

The reasonable demands of any textbook should be that the facts are right, that the essential matters under treatment are adequately covered, and that the presentation is lucid. This book largely meets these basic expect-

tations. The additional problem which an undergraduate text on psychopharmacology has then particularly to meet is the decision as to where the subject shall be circumscribed: neurophysiology, neuroanatomy, learning theory, clinical psychiatry, endocrinology, are among the obvious neighbouring states. In these borderlands it is difficult to find the balance between offering too much or too little contextual explanation and discussion, and in this regard the present text is not altogether successful. It is in these border areas that the student may be lost or misled. For instance, in respectable circles amphetamines are not these days prescribed for treatment of depression. Involutional depression is not a synonym for endogenous depression. More importantly, there is an invitation to sad unawareness in the declaration that "Our position is that mental (behavioural) disorders are the sole result of either learning or physiological dysfunction, or some combination of the two". Indeed, in one section a "simplistic overview" is boldly offered as a good thing.

This is in many ways an attractive book, but that lively seminar material has not been entirely successfully transmuted into a rounded textbook of psychopharmacology.

Griffith Edwards

Vector-parasite

Invertebrate Immunity: Mechanisms of Invertebrate Vector-Parasite Relations. Edited by Karl Maramorosch and Robert E. Schope. Pp. x+365. (Academic: New York and London, June 1975.) \$16.50; £7.90.

THIS is a collection of 24 papers by 29 authors given at a 'workshop' held in Bethesda, Maryland, in April 1974. It does not provide a complete or a balanced account of the present state of invertebrate immunology, but some of the contributions are useful as quarries for information or because they attempt a judicious review of particular topics.

Seven papers are grouped under the heading "Invertebrate Gut as a Barrier to Invading Parasites", an excellent subject, worthy of fuller discussion than it gets. A preliminary account of the midgut epithelium loses its way among electron micrographs of cell junctions, but T. C. Orihel shows how the peritrophic membrane of Diptera can limit infection by microfilariae and trypanosomes. All the other papers are concerned with viruses, particularly with factors affecting virus infection of the gut wall of vectors, principally insects. There is valuable material here for the specialist, but some of it is difficult to extract from the language. A surprising gap is the absence of any account of the fate of ingested bacteria.

"Analysis of Invertebrate Immunity" is not a appropriate heading for the next seven papers, some of which contain much that need not have been said again. An admirable contribution is that of R. S. Anderson, who reviews biochemical and other characteristics of phagocytosis by invertebrate cells *in vitro* and compares them with vertebrate systems. A. R. Barr discusses evidence for the genetical control of invertebrate (mainly mosquito) immunity to microfilariae and plasmodia.

Part III, comprising five papers on components of the haemolymph, includes a useful review by M. R. Tripp on humoral factors in molluscan immunity, and another by G. R. Wyatt on some biochemical features of insect and arachnid blood. J. S. Chadwick gives a detailed account of changes in the haemolymph related to infection or to induced immunity.

Another odd heading, "Vector Destruction", groups the last five papers. They include a largely pictorial account of encapsulation and melanisation in insects by A. J. Nappi, and a discussion by L. M. Riddiford of some of the available

evidence about hormones of insects in relation to their parasites.

Like most publications of its sort, this book is very uneven. Much that is important and useful is brought together in some of the contributions but the volume contains a lot of dead wood. Even specialists will have to use it circumspectly. The strong bias towards insects and molluscs as hosts and towards viruses, microfilariae and protozoa as parasites sorts ill with the claim that: "This book provides the first modern, integrated presentation of phenomena and mechanisms pertaining to immunity in invertebrate animals". All three adjectives can be challenged.

George Salt

Catalytic bodies

The Mathematical Theory of Diffusion and Reaction in Permeable Catalysts. By Rutherford Aris. Volume 1: *The Theory of the Steady state.* Pp. xvi+444. £13 net. Volume 2: *Questions of Uniqueness, Stability and Transient Behaviour.* Pp. xiv+217. (Clarendon: Oxford; Oxford University: London, 1975.) £8 net.

IN spite of the steady progress that is being made in improving the performance of catalysts, the problem of predicting the behaviour of a permeable catalyst, in which diffusion plays a role, remains an unpredictable and sometimes costly occupation. It is timely, therefore, that an excellent text on the mathematical theory, and its applications in chemical engineering and biology, should appear. Volume 1 deals comprehensively with the steady state behaviour of a catalytic body. Volume 2 establishes the links between uniqueness, stability and transient behaviour of reacting systems in which diffusion is involved.

Chapters 1 and 2 lay the physico-chemical basis of the theory and derive the general equations of diffusion and reaction, assuming the catalytic body to be a homogeneous continuum. This assumption has been called into doubt recently and there is a need for further experimental work to test the mathematical models.

Chapter 3 considers the single reaction in an isothermal catalytic body. There are two particularly interesting sections concerning the development of approximate solutions based on singular perturbation analysis and variational methods. Chapter 4 extends the study to non-isothermal behaviour. After a brief survey of numerical methods, the main theme, which deals with the complex interactions of diffusion and reaction, unfolds beautifully and we are left quite breathless with

the staggering prospect of possibly an infinite multiplicity of the steady state, although thankfully the conditions are rather extreme in practice. The concluding section on asymmetrical solutions is also potentially very important.

Chapter 5 is perhaps more low key but nevertheless focuses on the important area of multiple reactions. There is certainly much scope for ingenuity here in formulating non-uniform catalysts to improve yield. Chapter 6 deals with questions of existence and uniqueness of the steady state, followed by an outstanding discussion on stability in which important theoretical results are presented. The concluding chapter presents several fascinating aspects of the transient behaviour, such as deliberate periodic operation to improve selectivity, catalyst decay and so on.

The main objective of this book, which is to bring together many results on the theory of diffusion and reaction, is achieved with a style and grace which we have all come to recognise as pure Rutherford Aris. These two volumes should certainly be on the bookshelf of any aspiring chemical reaction engineer. His predictions may still be wayward but now at least he has the doubtful consolation of a hundred and one possible explanations.

D. L. Cresswell

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Structure-property relationships

Structure-Property Relations. By R. E. Newnham. (Crystal Chemistry of Non-Metallic Materials, Vol. 2.) Pp. x+234. (Springer: Berlin, Heidelberg and New York, 1975.) DM72; \$31.00.

THIS book essays a formidable task in its purpose of relating material properties and structure, in particular in demonstrating the application of some concepts in crystal chemistry to technologically important materials. The first chapter briefly reviews the principles of symmetry and crystal physics and emphasises the importance of such considerations in determining whether a given property can be developed by a crystalline material with a particular regularity of atomic architecture. In the remaining six chapters properties falling within the general fields of electronic transport, thermal properties and ion transport, ferroelectrics and other ferroic materials, optical materials, magnetic materials and materials with useful mechanical properties are described; in each case an outline of the physical principles on which the property is founded is given together with illustrations of particular structural configurations which promote the mechanisms of electronic and atomic movements responsible for the property.

To sketch in even the essentials of such a broad canvas in so short a volume demands economy of presentation. Newnham's text is compact, at times terse, so that it requires sustained concentration and careful study, even by a reader with some familiarity with the contents; this effort, however, often has its reward in an increased insight into a particular phenomenon. Inevitably, too, there must be occasions when the brevity demands simplistic statements which would be qualified in fuller and more rigorous accounts. These shortcomings are overcome by the references to other books and original papers which supplement each chapter and which must be accepted by a reader as an essential part of the presentation.

To assess the value of any book critically one should have a clear concept both of its objectives and the readership to whom it is directed, and it is on this latter aspect that doubts persist: the author's own preface provides no real clue, though perhaps there will be clarification when Volume 1 of the same series is published later. This book cannot be regarded as any kind of course text, though lecturers may find some parts stimulating when preparing their own course

presentation. To describe it as a review is probably misleading in that the limited coverage of any given topic requires that a practising scientist must go on to broader and deeper considerations. Its appeal is greatest if it is judged as some kind of hybrid between an advanced course text and a strict scientific review. In this light it could have a value on the library shelf for both inexperienced and experienced graduates, with sufficient background familiarity in materials studies, wishing to enter a new, unknown area of structure-property relations; but it must be emphasised most firmly that the character of presentation assumes a wide general knowledge of crystalline matter possessed by many materials scientists but certainly not by others, such as conventional engineers. Professor Newnham has made an honourable attempt to solve a difficult problem, but it may well be that a much more expanded treatment is essential; certainly it is a remedy that one is hesitant to recommend with the soaring costs of book production, already apparent in the price of this slim volume.

Peter Gay

Genetic combination

Genetic Structure and Function. By P. F. Smith-Keary. Pp. xvi+368. (Macmillan: London and Basingstoke, March 1975; distributed in the US and Canada by Halsted Press.) Boards, £7.95; paper, £3.95.

Genetic Structure and Function represents an attempt to encompass within a single volume an account of genetics at the molecular level, extending from classical genetic analysis to molecular biology. As the author observes in his preface, there are many books of general genetics, but they are all orientated towards the Mendelian approach, and the books on molecular genetics in general tend to start from a more biochemical position. This book is intended "to achieve a better balance between the genetic and the biochemical evidence".

After a brief introductory chapter, the next three chapters deal with the nature of the genetic material as nucleic acid, the structures of bacterial and viral genomes, and eucaryotic chromosomes. It is a straightforward account, the only deficiency of which is a failure to progress to the organisation of eucaryotic chromatin in terms of its components (such discussion is limited to a page).

The next group of chapters essentially concerns traditional genetic analysis in eucaryotes, fungi, and

bacteria and bacteriophages. The first of those chapters develops the principles of genetic mapping, a concern which is taken up in detail for each system in the succeeding chapters. This discussion is competently written if somewhat unexciting in view of the subject matter.

The remaining half of the book is devoted to what might be described as molecular biology, with chapters on the nature of mutation, the relationship between genes and proteins, the genetic code, transcription and translation and their control; a chapter on recombination is the only part of this which relates directly to the earlier material. This again is a well written and clear account, although necessarily of some brevity.

The final two chapters cover a variety of topics: suppression of mutations in bacteria, position effects in maize and *Drosophila*, gene expression in differentiation. These are the weakest; and the last chapter, given the broad title "Genetic Organization and Function in Eucaryotes", provides so condensed an account of its subject that it is difficult to believe readers will gain much from it. Discussion of the components of eucaryotic chromosomes and of the processes of transcription are perfunctory to say the least.

In view of the author's stated aim to provide a more balanced account of molecular genetics, perhaps it is surprising that the book commences with molecular descriptions, and then passes through classical genetic analysis to deal with molecular biology; a more logical development might have been to progress from a starting point of Mendelian genetics. As an introductory account, however, this book has much to commend it: it provides a complete discussion of all topics pertinent to procaryotic molecular genetics, in spite of its broad scope it is accurate and rarely oversimplified, it is well written and easy to follow, and the bibliography is sensible.

Dr Smith-Keary suggests that this book will be useful to second and third year undergraduates (in British terms): but one may feel that a more detailed account will usually be required by the third year. Indeed, given that molecular genetics is a subject of some sophistication, usually approached only after some knowledge of classical genetics has been attained, it is not surprising that separate books are usually written on classical and molecular genetics. The weaknesses of this book clearly are imposed by the attempt to provide a complete account covering such a breadth of material; one wonders if students would not in fact benefit from the more extended discussion possible when more than one book is used.

Benjamin Lewin

obituary

Vladimir Nikonovich Starovskii, a leading Soviet statistician and until recently Head of the Central Statistical Board of the Council of Ministers of the USSR, died on October 20 at the age of 70.

Starovskii, was born at Pomozdino, in what is now the Komi ASSR. At the age of 18, he enrolled at the University of Moscow, and, while still a student, began working for the Central Statistical Board. After graduating in 1926, and taking a postgraduate course at the Institute of Economics, he combined his duties at the Central Statistical Board with an academic career, holding a number of teaching and professorial posts, and publishing several major works on statistics, including *Parameters Characterising Correlation Connections*, *The Sampling Method*, *Theory of Mathematical Statistics*, and *Basic Problems of Population Statistics*. In 1940 he received the degree of Doctor of Economic Sciences, and in 1957 he was elected a Corresponding Member of the Academy of Sciences of the USSR. During his long career, Starovskii became known as one of the main organisers of governmental statistics in the Soviet Union. For his services in this field, he received numerous medals and prizes, including the Order of Lenin (three times), the Order of the Red Banner of Labour (twice) and the title of Hero of Socialist Labour.

Aleksandr Pavlovich Vinogradov, a vice-president of the Soviet Academy of Sciences, died on November 16, 1975.

Vinogradov was born on August 9 (Old Style), 1895 in St Petersburg. In 1920, he enrolled in the Military Medical Academy, and the Faculty of Chemistry of Petrograd University, graduating in 1925, to become an Instructor at the Military Medical Academy, and, simultaneously, an assistant at the Biogeochemical Laboratory of the Soviet Academy of Sciences. From

this time on, his career was closely associated with the Academy; he became a Corresponding Member in 1943, and a full member in 1963. From its foundation, in 1947, he was director of the "V. I. Vernadskii" Institute of Geochemistry and Analytical Chemistry of the Academy, and in 1967, he became one of the Academy's vice-presidents.

Vinogradov's numerous published works range from biology to geochemistry. He is particularly noted for his work on the distribution of elements in soils and bedrock, on the origin of ores, and for his hypothesis on the formation and evolution of the terrestrial crust and oceans. He also did important work on the geochemistry of the Moon and planets, and showed considerable interest in applications of atomic energy (in 1957 he read two papers to the Geneva Conference on the Peaceful Uses of Atomic Energy).

For twenty years, Vinogradov was head of the department of Geochemistry at Moscow State University, and is considered to be the founder of Soviet geochemistry. He was a winner of the Lenin and Stalin (now State) Prizes, and was awarded a number of high Soviet honours, including the Order of Lenin (three times), the Order of the Red Banner of Labour (twice) and various medals. **Vera Rich**

Torkel Weis-Fogh, Professor of Zoology at Cambridge, died on November 13 at the age of 53. He was a citizen of Denmark and a Fellow of the Royal Danish Academy, and before his election to the Cambridge chair he had been Professor of Zoophysiology at Copenhagen.

His first academic appointment was as assistant in research to August Krogh who had begun, after his retirement, a study of the metabolism of the desert locust. Weis-Fogh took up the flight mechanism and with the collaboration of Martin Jensen, an

engineer, he published several papers on the aerodynamics of locust flight. From aerodynamics he turned to the physiology of the locust flight muscle, and out of this interest in contractile systems was later to come the work, still in progress, on the vorticellid spasmoneme. Following up a difference between the apparent and the expected efficiencies of locust flight muscle he was led to a small pad of protein in the wing articulation, which he showed to have the properties of an almost perfect rubber. After coming to Cambridge in 1966 he went back to aerodynamics to investigate the flight of very small insects, whose ability to fly at all was unexplained, and out of this came the discovery of a hitherto unknown means of generating lift. These last two examples admirably illustrate his characteristic approach to a problem—analyse it and quantify it on the basis of current theory and then seek out the reasons for any anomalies.

Arising from his discovery of resilin, he became interested in the fine structure of the insect cuticle and the manner in which it is laid down, and also in the basis of the long-range elasticity of the vertebrate protein elastin. Yet he found time to spare from his own research to initiate important new inter-disciplinary enterprises. One of these was the use of electron microprobe X-ray spectrophotometry for the analysis of micron-sized areas in deep-frozen hydrated sections, a technique which will undoubtedly advance the work of many biological departments in Cambridge. And at the time of his death he was preparing, in collaboration with Sir James Lighthill, to launch a major project in biological fluid dynamics.

Weis-Fogh was held in the highest regard by zoologists and physiologists the world over, but nowhere more than in his own department where regard was compounded of affection as well as admiration. **J. A. Ramsay**

International meeting

January 8–9, **Food supplies: outlook for Britain**, UMIST Manchester (Royal Society of Health, Conference Department, 13 Grosvenor Place, London SW1X 7EN, UK).

Reports and Publications

Other countries

United States Department of the Interior: Geological Survey, Bulletin 1309: The Geologic Story of the Isle Royale National Park. By N. King Huber. Pp. v + 66. Water Supply Paper 2032: Ground Water in the Corvallis-Albany Area, Central Willamette Valley, Oregon. By F. J. Frank. Pp. ix + 48. (Washington, DC: Government Printing Office, 1974 and 1975.) [1310]

World Health Organization, Technical Report Series, No. 573: The Veterinary Contribution to Public Health Practice. Report of a Joint FAO/WHO Expert Committee on Veterinary Public Health. Pp. 79. Sw. fr. 8. No. 574: Pesticide Residues in Food. Report of the 1974 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. Pp. 37. Sw. fr. 6. (WHO: Geneva, 1975.) [1310]

United States Department of the Interior: Geological Survey, Bulletin 1387: Checklist of North American Late Paleozoic Coral Species (Coelenterata, Anthozoa). By William J. Sando. Pp. iii + 36. 70 cents. Water-

- Supply Paper 2035: Geohydrologic Reconnaissance of the Upper Potomac River Basin. By Frank W. Trainer and Frank A. Watkins, Jr. Pp. v + 68. Water-Supply Paper 2093: Quality of Surface Waters of the United States, 1968. Part 3: Ohio River Basin. Pp. x + 309. \$1.75. Water-Supply Paper 2121: Surface Water Supply of the United States, 1966-70. Part 7: Lower Mississippi River Basin. Vol. 2: Arkansas River Basin. Pp. ix + 931. \$6.15. Water-Supply Paper 2140: Ground-Water Levels in the United States, 1968-72. Northeastern States. Pp. v + 207. \$1.75. Water-Supply Paper 2147: Quality of Surface Waters of the United States, 1969. Part 8: Western Gulf of Mexico Basin. Pp. xi + 567. \$4. Water-Supply Paper 2156: Quality of Surface Waters of the United States, 1970. Part 7: Lower Mississippi River Basin. Pp. xii + 636. Professional Paper 858-B: Carboniferous Foraminifera and Algae of the Pennsylvanian of Wyoming. By Bernard L. Mamet. Pp. iii + 18 + 2 plates. Professional Paper 861: *Chesapecen*, a New Genus of Pectinidae (Mollusca: Bivalvia) from the Miocene and Pliocene of Eastern North America. By Lauck W. Ward and Blake W. Blackwelder. Pp. iii + 24 + plates 1-7. \$1.45. Professional Paper 868: Post-Paleocene Tertiary Rocks and Quaternary Volcanic Ash of the Wet Mountain Valley, Colorado. By Glenn R. Scott and Richard B. Taylor. Pp. iii + 15 + plate 1. \$1.55. Professional Paper 883: Discrimination of Rock Types and Altered Areas in Nevada by the Use of ERTs Images. By Lawrence C. Rowan, Pamela H. Wetlauffer, Alexander F. H. Goetz, Fred C. Billingsley and John H. Stewart. Pp. iv + 35. \$1.55. Professional Paper 900: Geological Survey Research, 1974. Pp. x + 349. Professional Paper 912: Petroleum Geology of Naval Petroleum Reserve No. 1, Elk Hills, Kern County, California. By J. C. Maher, R. D. Carter, and R. J. Lantz. Pp. viii + 109 + 25 plates. (Washington, DC: Government Printing Office, 1974 and 1975.) [1410]
- Journal of Molecular Catalysis*, Vol. 1, No. 1, September 1975. Edited by N. Boudart, V. Marconi, E. Kendall Pye and Ph. Teyssie. Pp. 1-76. Published bimonthly. Subscription price for 1975/76 (Vol. 1) Sfr. 140.00 including postage. (Lausanne: Elsevier Sequoia, S.A., 1975.) [1510]
- Drug and Alcohol Dependence*, Vol. 1, No. 1, September 1975. Edited by H. Halbach. Pp. 1-88. Subscription price for 1975/76 (Vol. 1): Institutskont. Sfrs. 115; Sfrs. 55. (Lausanne: Elsevier Sequoia, S.A., 1975.) [1510]
- Smithsonian Contributions to Zoology, No. 201: New Crayfishes (Decapoda: Cambaridae) from the Southern United States and Mexico. By Horton H. Hobbs, Jr. Pp. iii + 34. (Washington, DC: Smithsonian Institution Press, 1975. For sale by US Government Printing Office.) [1510]
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